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THE METABOLISM OF OYSTER SPERMATOZOA

by GEORGE FREDERICK HUMPHREY[1]

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(Accepted for publication 16th September, 1949.)

Reproduction of the Sydney rock oyster (*Saxostrea commercialis*, Iredale, 1936) involves external fertilization of eggs and sperm shed into the surrounding sea-water by the ripe organisms. During the spawning season the sexes are separate and each yields several million eggs or sperm. Stimuli to spawning may include the presence in the water of gametes of the opposite sex, certain algae, fluctuations in temperature, etc. An account of the life-history has been given by Roughley (1933).

After shedding the gametes are likely to encounter a non-stable environment, i.e. before fertilization actually occurs the environment may change in salinity, temperature, oxygen content, amount of dissolved organic matter, pH etc. Such alterations could affect the viability of the eggs or sperm by being beneficial or harmful in themselves. They could also produce changes (e.g. altered permeability) which might allow removal from the gametes of metabolic substrates or regulators, thus leading to different subsequent physiological responses in the eggs or sperm.

These questions are important in any consideration of problems arising from the normal reproductive process or methods of artificial propagation. These factors all have a biochemical basis, reflected in the case of spermatozoa by the phenomena of respiration, glycolysis, etc. A study of these reactions in oyster sperm is presented in this paper.

EXPERIMENTAL.

Material.

The animals used were adult oysters from the C.S.I.R.O. Experimental Farm at George's River, N.S.W. They were collected each week and kept in a shallow glass tank containing sea-water (salinity = 35‰) continually aerated by a fine stream of air-bubbles.

Glucose-1-phosphate, glucose-6-phosphate and fructose-1:6-diphosphate were prepared by the methods of Hanes (1940), Warburg and Christian (1932) and Neuberg and Lustig (1942). Commercial pyruvic acid was redistilled *in vacuo* and kept as a molar solution. Lactic acid was depolymerized before use by boiling a molar solution for a few minutes. Adenosinetriphosphate (ATP) was prepared by the method of Needham (1942), diphosphopyridinenucleotide (DPN) according to Williamson and Green (1940), lecithin from egg yolk by Pangborn's modification (1941) of the method of Levene and Rolf (1927), oxaloacetic acid

[1] This investigation was supported by a grant from the Research Committee, University of Sydney.

according to Fenton and Jones (1900) and α-ketoglutaric acid according to Friedman and Kosower (1946); glucose and fructose were obtained from Kerfoot and Co., England and other reagents from British Drug Houses.

Aerated sea-water was filtered by gravity through a grade 3 sintered glass funnel just before use.

Methods.

Preparation of Suspensions.

Oysters were sexed by low-power microscopic examination of small pieces of gonad tissue. Only the ripe males were used, i.e. those where fluid ran freely from the gonad on puncture with a scalpel and the sperm became highly motile after dilution with sea-water. All apparatus used for the preparation of sperm suspensions was rinsed in sea-water just prior to use. After sexing, the male gonad tissue was stripped over a dish containing sea-water; the tissue was stirred round and after a minute the mixture was allowed to strain through muslin. The creamy fluid was then centrifuged for 2 minutes at 1,500 r.p.m. to remove tissue debris and the supernatant carefully poured off. This was then centrifuged for 5 to 10 minutes at 3,000 r.p.m. to give good sedimentation of the majority of the sperm cells without packing them so hard that their motility was impaired. The sperm were then resuspended in sea-water by pouring gently from one tube to another. The suspensions were used immediately after preparation.

Counting of Sperm.

The sperm concentration was estimated after diluting 1 to 200 in a counting-pipette with 0·25 p.c. methyl violet; a Spencer bright-line haemocytometer with Neubauer ruling was used under high-power for the actual counting. The head of the sperm is about 2μ diameter and the tail about 40μ long.

Incubation Technique.

Anaerobic experiments were conducted in Thunberg tubes filled with N_2 freed from O_2 by passage over Cu turnings heated to 400° C. Measurements of gas exchange were made in Warburg manometers shaking at 96 cycles per minute using flasks of 10 ml. capacity; oxygen was determined in the presence of a centre-well strip of filter paper soaked in 20 p.c. KOH, respiratory quotients by Warburg's indirect method with flasks of 20 and 4 ml. capacity. Before analysis, experiments were terminated by the addition of trichloracetic acid to give a final concentration of 9 p.c.

Chemical Estimations.

The dry weight of suspensions was determined by heating at 70° C. overnight, a control being conducted simultaneously with an equivalent quantity of sea-water. Colorimetric determinations were carried out with a single-cell photometer using Hilger "spectrum filters," these estimations included citric acid (Krebs and Eggleston 1944), fructose (Roe, 1934), glucose (Folin and Malmros, 1929), pyruvic acid (Friedemann and Haugen, 1943), lactic acid (Barker and Summerson, 1941), phosphorus (Fiske and Subbarow, 1925) and total N (direct Nesslerization after combustion with H_2SO_4 and $HClO_4$). Glycogen was determined according to Humphrey (1941 a) except that the glucose was estimated as above. Protein was calculated as 6·25 times the total N figure. For the determination of phospholipide, the sperm suspensions or experimental mixtures were treated with an equal volume of 18 p.c. trichloracetic acid, centrifuged and the precipitate washed 3 times on the centrifuge with 9 p.c. trichloracetic acid. The residue was then extracted once with cold absolute alcohol and 3 times with alcohol-ether mixture (1 plus 3) at 60° C. The combined extracts were filtered,

evaporated to dryness and P determined after incineration with H_2SO_4 and $HClO_4$. Fatty acids were estimated by heating the sperm suspensions in the boiling water-bath for 30 minutes with excess alcoholic KOH and titrating with N/10 HCl using phenolphthalein as an indicator.

Results.

Composition of Spermatozoa.

Suspensions of sperm were washed again with 20 vol. of sea-water and the well-packed cells diluted with 7 vol. of sea-water. Calculating the phospholipide as lecithin and the fatty acids as stearin, ripe sperm contained, on a dry-weight basis (89 p.c. of the fresh weight is H_2O), 52 p.c. protein, 7·9 p.c. stearin, 0·84 p.c. lecithin and 0·82 p.c. glycogen. Figures collected over only two spawning seasons suggest that the chemical composition changes as summer progresses but that protein and fat are always the major components. Sperm and gonad tissue do not contain citric acid and only small (up to 20 mg. p.c.) amounts of fructose.

Factors Affecting Normal Respiration.

Temperature. Although sperm suspensions consumed O_2 at 37° C., motility was quickly lost. At 25° C., respiration proceeds and motility is maintained with little diminution for several hours. The range of summer temperature of the water in which the animals were grown was 18 to 25° C. All experiments were therefore conducted at 25° C.

pH. The optimum pH for oxygen uptake occurred over a fairly wide range (7 to 8). The value chosen for the experiments was 7·5. Table 1 gives the effect of varying the pH between 6 and 8. At pH 6·0 and 6·5 the respiration almost ceased after 10 minutes. At the other values, oxygen consumption proceeded uniformly throughout the experimental period. The oxygen uptake is depressed if the concentration of phosphate in the suspending medium is greater than 0·004 M.

TABLE 1.

Effect of pH on oxygen consumption.

The sperm concentration was 8 × 10^8/ml. Each flask contained 1 ml. suspension + 0·1 ml. 0·04 M Na_2HPO_4–KH_2PO_4 buffer to give the final pH stated (checked by glass electrode).

pH	µl. O_2/40 min.
6·0	12
6·5	24
7·0	59
7·5	55
8·0	55

Centrifugation. Prolonged centrifuging, shaking or stirring with a glass rod all have an adverse effect on respiration. Separation on the centrifuge is not so easily accomplished with oyster sperm as with some other types (e.g. ram) owing to a tendency to form stringy masses; if these occurred they were discarded in the mixing process. In Table 2, whole suspension refers to the gonad extract in sea-water after passage through muslin; centrifuged suspension to the sperm prepared in the normal manner; and supernatant to the few cells which do not centrifuge down. The addition of glucose to the various fractions did not alter the respiration rate.

TABLE 2.

Oxygen consumption of sperm.

1 ml. sperm in each flask; the figures given are μl. O_2/hr.

	/10^8sperm	/mg.N.
Whole suspension (6×10^8sperm/ml.)	1·3	12
Centrifuged suspension (8×10^8sperm/ml.)	2·7	22
Supernatant suspension ($0\cdot2\times10^8$sperm/ml.)	5·0	3

The results indicate that the whole suspension and the supernatant contain much nitrogenous non-respiring matter and that this is not present to such an extent in the centrifuged suspension. The higher Z_{O_2} (μl. O_2/hr./10^8 cells) of the supernatant must be attributed, not to any properties of the sperm cells isolated in this particular fraction, but to a dilution effect (see below).

Concentration of sperm. Experiments on the relation between respiration and the density of the suspensions were carried out for 3 hour periods. Linear oxygen uptakes were observed with densities of 5·5 to 53 $\times$ 10^8 sperm/ml. The best retention of motility was found in the concentration range of 6 to 12×10^8/ml. Extensive dilution of the suspensions gave larger values for Z_{O_2} (Table 3).

TABLE 3.

Effect of dilution on respiration.

1 ml. of sperm suspension in each flask.

Z_{O_2}	Sperm density ($\times10^8$/ml.)
1·6	20
1·9	10
4·0	1
2·0	0·2
100	0·04

For subsequent work the final concentration of sperm in experimental mixtures was kept in the range $5–20\times10^8$ cells/ml.

O_2 and CO_2 tensions. Gassing the manometer flasks with pure O_2 gave only a slight increase in respiration. Also, when the gas phase was 1 p.c. CO_2 in O_2 and the suspending fluid contained $NaHCO_3$ to keep the pH at 8·0, the respiration and persistence of motility were only slightly increased.

Under the conditions of experiment the intensity of respiration in the absence of added substrate, varied from about 2–10 μl. O_2/10^8 cells/hr. and 3–4 μl. O_2/dry mg./hr. On the basis of each mg.N the respiratory rate was rather constant at about 20 μl. O_2/hr.

Substrates for Respiration.

The compounds tested were dissolved in sea-water to give 0·1 M solutions (5 p.c. in the case of glycogen and a 0·05 M suspension in the case of lecithin), and the pH adjusted to 7·5. Oxaloacetic and α-ketoglutaric acids were made up immediately prior to pipetting the sperm suspension. Added phosphate (final concentration = 0·004 M) did not affect the results.

TABLE 4.

Substrates and oxygen consumption.

1 ml. sperm suspension + 0·1 ml. substrate in each flask; the figures given are p.c. change over a 3 hour period.

Increase		No effect or only slight increase	Decrease	
α-ketoglutaric acid	150	glycogen	acetic acid	35
oxaloacetic acid	150	glucose	propionic acid	15
pyruvic acid	20	fructose	butyric acid	10
glycine	15	lactic acid	formic acid	10
		succinic acid		
		fumaric acid		
		malic acid		
		glutamic acid		
		lecithin		

Thus, some compounds which serve as respiratory substrates for mammalian sperm (Humphrey and Mann, 1949), are without effect (glucose, fructose, etc.) or inhibit (fatty acids). Analyses of the flask contents after the experimental period showed that, with fructose and glucose as substrates, no significant disappearance of these compounds had occurred; nor was there any loss under anaerobic conditions (incubation for 2 hours in Thunberg tubes). However, in the case of lecithin, although there was no increased respiration there was a small loss of phospholipide P (250 μg.P decreased to 235 μg. on incubation for 4 hours with 19×10^8 sperm). This metabolism of phospholipide was confirmed by analysis of sperm suspensions before and after incubation with no added substrate; here the loss of phospholipide P averaged 0·1 μg./10^8 sperm/hr.

Dehydrogenases.

The method of Quastel and Wheatley (1938) was used to estimate the total content of dehydrogenase systems which would react with $K_3Fe(CN)_6$. The experiments (Table 5) were carried out in an atmosphere of 1 p.c. CO_2 in N_2, the flask contents containing $NaHCO_3$ to give a pH of 8·0.

TABLE 5.

Presence of dehydrogenases.

5 × 10^8 sperm in 1 ml. flask contents; when added, 1·0 mg. DPN was used in each flask. Final concentration of each substrate was 0·01 M. The results are calculated for the first hour.

	Q_{CO_2}
No addition	7
Glucose	9
Succinic acid	7
Malic acid	7
Malic acid + DPN	8
Lactic acid	7
Lactic acid + DPN	7

Tests made by the Thunberg technique showed that the sperm possessed active mechanisms for reducing methylene blue and that this action was hastened by the presence of 0·01 M succinate; 9×10^8 sperm could reduce 0·1 ml. of 0·015 M methylene blue in 120 minutes after the addition of succinate.

Glycolysis.

Freshly prepared sperm suspensions contained about 1 μg. lactic acid/10^8 sperm, and no pyruvic acid. On incubation with various substrates there was very little formation of lactic acid and no pyruvic acid at all was produced (Table 6). There was no difference in glycolytic rate under aerobic or anaerobic conditions; neither 0·05 M nicotinamide nor 0·2 mM fructose-diphosphate accelerated the reactions.

TABLE 6.

Glycolysis.

15×10^8 sperm incubated in N_2 or air for 2 hours, in the presence of 0·01 M phosphate, 0·005 M $MgCl_2$, 0·001 M ATP, 0·002 M DPN, 0·002 M nicotinamide and 0·01 M substrate (or 0·5 p.c. glycogen). Final volume = 1 ml.

Substrate	Z_L (μl. lactic acid/10^8 sperm/hr.)
—	0·04
Glycogen	0·02
Glucose	0·01
Fructose	0·05
Glucose-1-phosphate	0·04
Glucose-6-phosphate	0·06
Fructose-diphosphate	0·30

Activators and Inhibitors of Respiration.

Sperm suspensions give positive nitroprusside reactions for –SH groups and the importance of these is shown by the fact that reagents such as iodoacetic acid (10^{-3} M) inhibit oxygen consumption; however, very low concentrations, 10^{-6} M, stimulate respiration. Addition of compounds containing –SH linkages increases metabolism.

TABLE 7.

Effect of activators and inhibitors on respiration.

$13 \cdot 5 \times 10^8$ sperm in 1 ml. flask contents containing 0·004 M phosphate; the figures relate to the first hour (control = 100).

	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M
Phenylmercuricacetate	—	—	25	80	100
Iodoacetate	—	16	78	108	125
Arsenite	—	6	33	122	—
Azide	—	50	67	90	—
Cyanide	—	4	5	22	—
Methylene blue	—	—	180	116	108
Brilliant cresyl blue	—	—	185	148	105
Glutathione	205	—	—	—	—
Cysteine	183	—	—	—	—

Further, the absorption bands of cytochrome can be seen under the microspectroscope and cytochrome oxidase inhibitors such as cyanide and azide both inhibit oxygen uptake by sperm. A positive benzidine $-H_2O_2$ reaction is obtained with sperm suspensions.

Cyanide did not affect the motility of the sperm cells whereas compounds such as phenylmercuricacetate abolished or lowered the activity. When respiration was being inhibited by the various reagents there was an increase in glycolysis (Table 8).

TABLE 8.

Effect of respiratory inhibitors on glycolysis.

$13{\cdot}0\times10^8$ sperm in 1 ml. flask contents containing 0·004 M phosphate; incubated for 240 minutes.

	μg. pyruvic acid	μg. lactic acid
0 min.	0	7
240 min.	0	9
+ 0·001 M iodoacetate	9	20
+ 0·001 M cyanide	3	15
+ 0·0001 M phenylmercuricacetate	5	14

Catalase.

Incubation of sperm suspensions with H_2O_2 gave an evolution of gas and a disappearance of H_2O_2, as judged by titration with $KMnO_4$. Using H_2O_2 at a final concentration of 0·1 M and terminating the reaction at 10 min. with $5N.H_2SO_4$ the $Z_{H_2O_2}$ (μl. H_2O_2 decomposed/10^8 sperm/hr.) was about 3,000.

Respiratory Quotient.

The R.Q. was determined over a period of 30 minutes. The values varied between 0·90 and 1·05. During this period also, there was no formation of acid as judged by the initial and final bicarbonate concentration of the medium.

DISCUSSION.

Chemical Composition.

In common with other lamellibranchs, oysters are distinguished by a high content of glycogen; in *S. commercialis* as much as one-third of the dry weight may be composed of this substance (Humphrey, 1941 b). The sperm of this species however, contains less than 1 p.c. glycogen on a dry-weight basis. This figure is similar to that given by Mann (1946), who states that the glycogen content of ram semen is usually about 0·1 p.c. Stott (1930) gives the value for *Echinus esculentus* sperm as 0·2 p.c. of the wet weight. The dry weight of 10^8 oyster sperm was 1 mg.; in comparison, figures for 10^8 sperm from other species are: guinea-pig 5 mg. (Redenz, 1933), bull 3 mg. (Redenz, 1933) and ram 3 mg. (Mann and Lutwak-Mann, 1948). The absence of citric acid and the small quantity of fructose (including fructose-phosphates) differentiates this species from organisms whose male reproductive tissues contain both

(ram, bull, etc., Humphrey and Mann, 1948; opossum, Humphrey and Robertson, unpublished) or only one of these compounds e.g. fructose (grasshopper, Humphrey and Robertson, 1949). The amount of phospholipide present is about one-tenth of the value given for ram sperm by Lardy, Hansen and Phillips (1945).

Preparation of Spermatozoa.

Methods used for the preparation of sperm suspensions are essentially a compromise between washing the cells free from possible substrates, salts, interfering substances, etc., and keeping the sperm in an undamaged condition. The small size of oyster sperm as compared with mammalian sperm makes centrifugation more difficult, but the apparently greater impermeability of oyster sperm means that there would be less uptake of foreign compounds from tissue fluids set free when the gonad is ruptured. However, the presence of nitrogenous material in the tissue fluid would vitiate Z_{O2} values based upon a mg. N basis; for this reason figures have been calculated on the numerical basis of 10^8 sperm cells. This point has been raised by Rothschild (1948 c) with regard to suspensions from *Echinus esculentus*, the seminal plasma of which contains significant quantities of protein.

The pH of normal sea-water is about 7·5–8·2 and it is not surprising that this range is also the optimum one for respiration and motility. Carter (1931) and Rothschild (1948 c) have made similar observations for sea-urchin sperm.

The increase in respiration on dilution (Table 3) is a physiological phenomenon and not a result of maltreatment of the sperm during preparation. Gray (1928) has made an extensive study of this phenomenon ("Dilution Effect") for sea-urchin sperm and concludes that the lack of motility in concentrated suspensions (e.g. in undiluted testicular fluid) is probably due to the physical effect of crowding; the total energy expenditure during the life of the sperm cell depends on the degree of subsequent dilution when the sperm is shed into the surrounding sea-water or when suspensions are made under laboratory conditions. Rothschild (1948 c) showed that the dilution effect is not due, among other things, to CO_2 accumulation or to dilution of inhibitors in the medium or released from the sperm cell itself. For some species, the importance of keeping the sperm concentration within definite limits is shown by the observation of Mann and Lutwak-Mann (1949) that dilution of suspensions of ram sperm from 6·5 to 1·3 $\times$ 10^8/ml. causes the rates of glycolysis and respiration for each sperm cell to be decreased by about 50 p.c. It is possible that the lack of this phenomenon in oyster sperm is related to the fact that in normal shedding of the sperm into the sea, large dilution occurs, stimulating the metabolic processes so that there is a consequent increased energy production allowing more movement to be made before fertilization occurs.

Metabolism.

With the concentration of sperm used, increasing the oxygen content of the gas phase to 100 p.c. had little effect on the amount of oxygen consumed. This is in agreement with the findings of Rothschild (1948 c) on sea-urchin sperm and Humphrey and Mann (1949) on ram sperm. Unlike ram sperm, the respiration of the oyster sperm was not increased by the presence of extra $NaHCO_3$ in the medium and CO_2 in the gas-phase. Barron (1932) has shown that in some species (sea-urchin and starfish) the motility and fertilizing capacity of sperm are lost after 1–2 hrs. in N_2 but that cyanide "anaerobiosis" prolongs the life of the cells. Similar findings were obtained with the oyster since sperm kept in Thunberg tubes filled with N_2 quickly lost their motility but regained this to a slight extent when re-exposed to air; suspensions kept in 0·001 M cyanide preserved the same motility as in air. Neglecting the possibility that exposure to a partial vacuum (about 30 mm. Hg.) exerts a harmful effect, it does seem that "physical" lack of oxygen is harmful although "chemical" lack is not.

The intensity of respiration of oyster sperm (Q_{O2} = 3 to 4)is about half that of ram sperm (Q_{O2} = 8; Mann, 1945 b) and about the same as sea-urchin sperm (Rothschild, 1948 a); human sperm (Z_{O2} = 1 to 2; MacLeod, 1943) have a lower rate than oyster sperm (Z_{O2} = 2 to 10). The question then arises as to what is the substrate for this endogenous oxygen consumption. Determinations of respiratory quotient cannot be taken as furnishing unequivocal proof of the combustion of protein, fat or carbohydrate; R.Q. values for ram sperm (Humphrey and Mann, 1949) do indicate that after carbohydrate has been exhausted, fat could provide the substrate. Over shorter periods the values of the R.Q. found for other species are 0·92 for fowl sperm (Winberg, 1939), 1·08 for sea-urchin sperm (Barron and Goldinger, 1941) and 0·9 to 1·0 for oyster sperm. These figures give no idea as to the nature of the substrate used when the period is prolonged. Analytical methods give much more reliable indications and Lardy and Phillips (1941 b) have shown that when bull sperm are stored there is a drop in the phospholipide content, about 1μg. lipide P being used/10^8sperm/hr. This is 10 times the rate at which oyster sperm metabolise phospholipide. Lardy and Phillips (1941 a) have also shown that added phospholipide can increase the respiration of bull sperm, thus strengthening the case for phospholipide being a natural endogenous substrate which can be used in the absence of carbohydrate. However, attempts to demonstrate similarly increased respiration with oyster sperm were unsuccessful; it is not possible to conclude that phospholipide is not a natural endogenous substrate because the added phospholipide may not have penetrated into the sperm. Rather, this lack of effect might be taken as supporting the case for phospholipide because if permeability to phospholipide is a purely physical process, ease of

escape would be a natural consequence of ease of penetration and this would mean that when the sperm were shed into the sea the substrate would be lost. Similar considerations apply with equal force to utilization of other compounds.

In the case of succinic acid it is possible to state that this can penetrate the sperm since the time of reduction of methylene blue is shortened by this compound, but no increased oxygen consumption is caused by adding succinic acid to respiring sperm suspensions. Of course, the concentration of succinate needed inside the sperm to accelerate methylene blue reduction may be less than that required to increase the oxygen uptake and therefore succinic acid could still be a normal metabolite. Human (MacLeod, 1943) and sea-urchin sperm (Barron and Goldinger, 1941) show increased oxygen consumption in the presence of succinic acid whereas ram sperm (Humphrey and Mann, 1949) does not; succinic acid does not preserve motility in human sperm although it does increase oxygen uptake and also accelerates the reduction of methylene blue.

Glycolysis.

When compared to the glycolytic rates observed in other sperm ($Z_L{}^{N_2}$ = 7 to 42 in the presence of glucose for ram sperm, endogenous values being about one-tenth of this figure, Mann, 1945 b) it seems that only negligible glycolysis occurs with oyster sperm. However, in view of the uncertain knowledge of the permeability of the sperm this conclusion must be applied only to the question of acid-production from endogenous substrates; in this case, glycolysis is certainly very feeble ($Z_L < 0{\cdot}1$). The results in Table 8 indicate that acid-production may be of some importance in metabolism because when respiration was inhibited, there was an increase in the production of pyruvic and lactic acids; the amounts formed, however, were still very small. In sea-urchin spermatozoa, which have no anaerobic or aerobic glycolysis, suppression of respiration with CO does not yield any acid-production as determined manometrically by displacement of CO_2 from $NaHCO_3$ (Rothschild, 1948 c). In ejaculated bull sperm, which shows high glycolytic rates (Lardy and Phillips, 1943 a) respiratory inhibitors have a variable effect on glycolysis; cyanide increases glycolysis whereas azide and iodoacetate inhibit.

Inhibition of Respiration.

Compounds which destroy –SH groups and others which attack cytochrome oxidase are all powerful inhibitors of the respiration of oyster sperm (Table 7). However, as Rothschild (1948 c) has pointed out respiration can be reduced in a number of cases without motility being affected; the relationship between movement, ability to respire and ability to reduce dyes such as methylene blue remains obscure. It seems that ability to respire can be lost first, then motility but that dehydrogenase activity still persists. The demonstration by

Zittle and Zitin (1942) of cytochrome oxidase in bull sperm, and the spectroscopic observations of various cytochrome components in *Arbacia* sperm (Ball and Meyerhof, 1940) and in ram sperm (Mann, 1945 a) confirm the importance of the cytochrome system in the metabolism of this type of cell as suggested by the effects observed with inhibitors. In comparison with these species, oyster sperm has a much lower content of cytochrome, the absorption bands being definite but never very strong; washed suspensions of oyster eggs have quite a pink tinge and the absorption bands of cytochrome are seen very readily.

The work of Barron, Nelson and Ardao (1948) and of Lardy and Phillips (1943 b) showing that very low concentrations of –SH reagents accelerate respiration whereas higher concentrations give almost complete inhibition, was confirmed in the present study with oyster sperm. Barron *et al.* interpret these findings as showing that cell metabolism is regulated by the soluble –SH groups (those which react with very low concentrations of –SH reagents) and that when these are removed respiration increases; with higher concentrations of –SH reagents the thiol groups of essential proteins are destroyed and inhibition of metabolism results. If these suggestions apply to oyster sperm it is difficult to reconcile them with the finding that with this species, respiration is stimulated by soluble –SH groups (cysteine, glutathione).

Catalase.

Gonad tissue in the oyster is richer in catalase than any other part examined (Humphrey, 1943). The occurrence of significant quantities of this enzyme in sperm has not yet been shown for other species; Rothschild (1948 b) states that some is present in the seminal fluid of *Echinus esculentus*. Under certain conditions H_2O_2 accumulates as a result of sperm metabolism (i.e. oxidation of tyrosine, tryptophane or phenylalanine) and can be toxic (Tosic and Walton, 1946; Tosic, 1947).

Conclusions.

The results on the effect of temperature and pH on metabolism show that oyster spermatozoa are adapted to the conditions they are likely to encounter when they are shed into the surrounding sea-water.

The rate of oxygen consumption is higher than for other tissues of this species and this is correlated with the lack of glycolysis exhibited by the sperm i.e. these cells depend on a purely oxidative type of metabolism. In spawning periods, when there is a quantity of both sperm and eggs in the water there would be a localized depletion of dissolved oxygen; it is not possible at the moment to say what the extent of this would be nor if it would have adverse effects on the life of the gametes.

SUMMARY.

Suspensions of active spermatozoa can be prepared from the oyster by a process involving gentle centrifugation. The optimum pH for respiration is about 7·5; concentrations of phosphate higher than 0·004M depress oxygen consumption. Extensive dilution of suspensions (below 6×10^8/ml.) caused rapid loss of motility.

Increased respiration was caused by α-ketoglutaric, oxaloacetic, pyruvic and aminoacetic acids; acetic, propionic, butyric and formic acids depressed respiration. Lactic, succinic, fumaric, malic and glutamic acids, glycogen, glucose, fructose and lecithin had only insignificant effects. During respiration there was a loss of phospholipide.

There was only slight glycolysis in the presence of a number of compounds, the highest rate (0·30 μl. lactic acid/10^8 sperm/hr.) being observed with fructose diphosphate.

Respiration is inhibited by agents which combine with cytochrome oxidase or –SH groups. In very low concentrations –SH reagents exert a stimulating effect. Under conditions where respiration is inhibited there is a slight increase in glycolysis.

Catalase can be detected in sperm suspensions.

The results are discussed in relation to the physiology of reproduction in the oyster and other animal types.

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STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

I. THE STATE OF VIRUS RECEPTORS AND INHIBITORS IN THE RESPIRATORY TRACT

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This study is the first of a series initiated to investigate the separate mechanisms possibly involved in infection by and resistance to influenza viruses. By making full use of all available techniques, most of them recently developed, it was hoped to follow the sequence as a whole, so as to derive an expression which would generalize all the information we already have.

The response of the animal organism to influenza virus, live or inactivated, was studied in mice. Although by no means identical with epidemic influenza, experimental infection of the mouse closely parallels the corresponding human condition, the differences being quantitative rather than qualitative. With this reservation, the mouse is still the standard animal for experimental studies and for the assessment of vaccines, as is the guinea-pig for tuberculosis or diphtherial infection—a model allowing the essential pathological and immunological processes to be elucidated. Yet, apart from investigations on the degree of lung lesions in relation to dose, no comprehensive study of the whole process of infection and immunity has been made.

Before commencing a systematic study of the specific immunological response, it seemed desirable to determine the participation and possible rôle of nonspecific components (viz. the behaviour of receptors on the susceptible cells and of inhibitors in the respiratory tract) during the process of infection or immunization. The importance of cellular receptors in relation to infection was clearly demonstrated by the work of Stone. In her comprehensive studies on the biological activities of RDE, the "receptor destroying enzyme" of *V. cholerae*, she established the principle of minimum receptor requirements (Stone, 1947 b, 1948 a, b). Susceptibility to the viruses of the mumps-influenza group is conditioned by the state of receptors on the future host cell, each strain having a characteristic level below which infection cannot be initiated. Destruction of receptors equals prophylaxis against infection, this being the mechanism of RDE-action in preventing infection both in embryonated eggs and mice.

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In view of the surprisingly close correlation between the number of available receptors as tested in the excised lung (Fazekas de St.Groth, 1948 b), and the susceptibility to infection of animals treated with RDE (Stone, 1948 b), a quantitative technique was provided by which the state of virus receptors could be determined at any phase of the disease or recovery. The changes occurring in the number of available receptors and in the virus inhibitors of the respiratory tract were therefore studied after experimental infection of mice with graded doses of influenza virus. The findings are correlated with each other and with the pathological changes occurring in infected lungs. Similar experiments with killed virus, or active virus given by routes other than the nasal are included.

MATERIALS AND METHODS.

Normal saline. 9·00 gm. NaCl in 1,000 ml. distilled water.

Citrate saline. 20·00 gm. sodium citrate and 9·00 gm. NaCl in 1,000 ml. distilled water.

Horse serum saline. 10 p.c. horse serum and 90 p.c. normal saline, sterile.

Red cells. Venous blood of adult fowls was collected in citrate saline. The cells were washed twice and finally made up to a 1 p.c. suspension in normal saline. No cells older than 36 hours were used in the experiments.

As is the routine of this Institute, the blood of different fowls was not pooled, and only the most suitable cells were used for special purposes. Thus "inhibitor insensitive" cells were used for the titration of haemagglutinin (virus), and "inhibitor sensitive" cells for inhibitor titrations (Anderson, 1948). Both types of cells had to be "lipid insensitive," i.e. non-agglutinable by lipid agglutinins (Stone, 1946). This precaution was taken because both bronchial washings and the saline extract of ground lungs contain lipid agglutinin which might interfere in the assay of either virus or inhibitor.

Virus.

The Melbourne (influenza A) and the LEE (influenza B) strains were used in the experiments. The viruses were grown in the allantoic cavity of hen's eggs by the method of Burnet. The fluids were harvested 42 hours after inoculation. Those free from bacterial contamination, and of titres higher than 500 were pooled and stored at 4° C.; they are referred to in this paper as "active." No fluid older than seven days was used in the experiments.

Receptor Destroying Enzyme (RDE).

The "4Z" strain of *V. cholerae* was grown on soft (1 p.c.) agar plates at 37° C. for 16 hours. The agar was collected into a gauze bag and pressed through a Büchner funnel. The filtrate was left to settle in the refrigerator, and the supernatant removed. This fluid was heated in the presence of excess calcium at pH 6·0 at 55° C. for 40 minutes, and the precipitate removed by centrifugation. The yellowish, completely transparent fluid so obtained had a titre of 2,000, as estimated by the standard technique. It will be referred to as RDE. For full details of its preparation and properties see Stone (1947 a) and Burnet and Stone (1947).

Antisera.

Anti-virus serum was obtained from ferrets experimentally infected with virus. 0·25 ml. of a 1:100 dilution of suitable allantoic fluid was given intranasally, and the animals were killed 12 days after infection. Merthiolate (1:10,000) was used as preservative, and the sera were stored at 4° C.

Titration of Virus Haemagglutinin.

Because of the presence of RDE in some samples, the titrations were done in citrate saline. As haemagglutinin titres are not altered in this diluent while the action of RDE on receptors is greatly reduced, 10 AD of virus could be accurately assayed in media containing 1,000 units of RDE.

Doubling dilutions were made in 0·25 ml. citrate saline and an equal volume of 1 p.c. fowl cells added. The pattern of settled cells was read after an hour's standing at room temperature. 1 AD is the amount of virus which causes partial ("+") agglutination of 0·25 ml. 1 p.c. fowl cells. The titres are expressed as the reciprocal of the dilution giving partial agglutination.

Inhibitor Titration.

The sample was diluted in two-fold steps in 0·25 ml. volumes of normal saline. One standard drop (approximately 0·04 ml.) of heated LEE virus (30 minutes at 56° C.) containing 5 agglutinating doses was added, and the racks left at room temperature for 30 minutes. At the end of this period 0·25 ml. of 1 p.c. inhibitor-sensitive fowl cells was added, and the test read after a further 60 minutes. Partial agglutination is taken as the end-point.

The technique is a modification of those proposed by Anderson (1948) and Burnet (1948 b); for the principles underlying the method see the quoted papers.

Absorption of Virus.

As the bronchial washings in which the inhibitor was to be determined occasionally contained active virus capable of destroying this inhibitor, 10 p.c. washed, packed human red cells were added to the samples immediately after they had been taken. As active virus has a higher affinity for red cells than for the inhibitor, it was possible by this means to adsorb it to the cells. After 20 minutes at room temperature the tubes were spun and the supernatant removed. As determined in control experiments, this treatment removed all haemagglutinin without altering the inhibitor content of the fluids.

Experimental Animals.

Healthy 6-7 weeks old laboratory mice of uniform breed were obtained from the Institute's animal rooms. Both sexes were represented in all experimental groups. *Intranasal instillation* of 0·05 ml. volumes of fluid was done under light aether-chloroform (2:1) anaesthesia. *Intraperitoneal injections* of 0·10 ml. volumes were given from a tuberculin syringe in the right suprainguinal region. The site of *subcutaneous inoculations* was the loose connective tissue of the lumbar region.

Preparation of Mouse Lungs.

Under aether-chloroform anaesthesia the animals were bled out from their art. subclavia. The ribs were cut along the costo-cartilaginous line and the anterior wall of the thorax removed. Dissection of the trachea-oesophagus-lung complex was begun close below the larynx. Any damage to the lungs was carefully avoided. The trachea was slipped over a glass cannula inserted in a one-hole rubber stopper, and fastened to it by a thread. After rinsing the pleural surface of the lungs, the stopper was placed in a large test tube with a side arm through which the pressure inside the vessel could be regulated. If fluid is put in the cannula and the pressure lowered inside the vessel, the excised lung will aspirate it; by appropriate blowing through the side arm it can be expelled again. By this method the respiratory surface can be washed at suitable intervals and the washing fluids tested.

A detailed description of the method is given in Fazekas de St.Groth (1948 a).

Assay of Available Receptors.

When allantoic fluid containing virus is introduced into the excised lungs the virus is adsorbed to the respiratory lining, and subsequent washings at first contain no detectable haemagglutinin. After a certain time active virus elutes and measurable amounts of agglutinin appear in the washings. If the total amount of agglutinin regained in the washing fluids is plotted against time, a characteristic adsorption-elution curve is obtained. It is sufficient for all practical purposes to use 10 washings in an experiment of three hours.

In the actual tests the lungs were washed with three changes of 0·50 ml. normal saline and—to neutralize traces of residual virus in experiments on infected lungs—with 0·5 ml. dilute specific antiserum sufficient to bind 500 AD of the virus used for infection. Then the standard amount of virus was added and kept in the lungs for 10 minutes. As the Melbourne strain of influenza A was used to infect the mice, in the assay of receptors 0·50 ml. of LEE (B) virus was used. 640 AD were aspirated, an amount whose 50 p.c. elution value (T_{50}) is 150 minutes. The respiratory tract was washed with 0·50 ml. volumes of normal saline five times in the first hour, three times in the second and twice in the third. To check the quantity of virus used, the lungs were treated with dilute RDE for 15 minutes after the completion of the experiment, and thus all of the virus was removed.

The method is fully described and discussed in a previous paper (Fazekas de St.Groth, 1948 b).

EXPERIMENTS.

The State of Respiratory Receptors after Infection.

A large number of mice was inoculated intranasally with dilutions of active Melbourne virus. Groups of six mice were killed at twenty-four hour intervals following the infection, up to the twentieth day. Their lungs were excised and tested for available receptors by the technique described under Methods. As shown in a previous study (Fazekas de St.Groth, 1948 b), the number of receptors can be satisfactorily measured by the time in which half of a known virus dose elutes from the lungs under standard conditions. This value, T_{50} shows very little variation in normal lungs and the accuracy of the technique is limited only by the accuracy of the haemagglutinin titrations. For the sake of easier comparison the T_{50} values are expressed as percentage of the 50 p.c. elution time in normal lungs. Thus "$T_{50} = 100$" means "number of receptors as in normal lungs"; values above 100 indicate an increase in available receptors, those below 100 a decrease.

Fig. 1 gives the result of a typical experiment. The mice were infected with a 1:100,000 dilution of MEL virus, an amount which causes an average of 50 p.c. lung consolidation in seven days. Each point represents the arithmetic mean of six lung-experiments. The averages of two normal lungs on each day of the test are included as controls.

The 50 p.c. elution time, that is the number of available receptors, does not change significantly in the first four days after infection. From then on there is a progressive decrease and the T_{50} value reaches its minimum on the seventh day. It should be noted, however, that even the smallest number of receptors is well above the "critical level", the minimum necessary for infection with any strain of influenza virus. Between the seventh and eleventh days the number of receptors increases in a typically hypercompensative fashion: T_{50} values between the tenth and fourteenth days are definitely above the normal. As will be seen below, this hypercompensation is even more marked after infections with heavier inocula of virus when the previous destruction of recep-

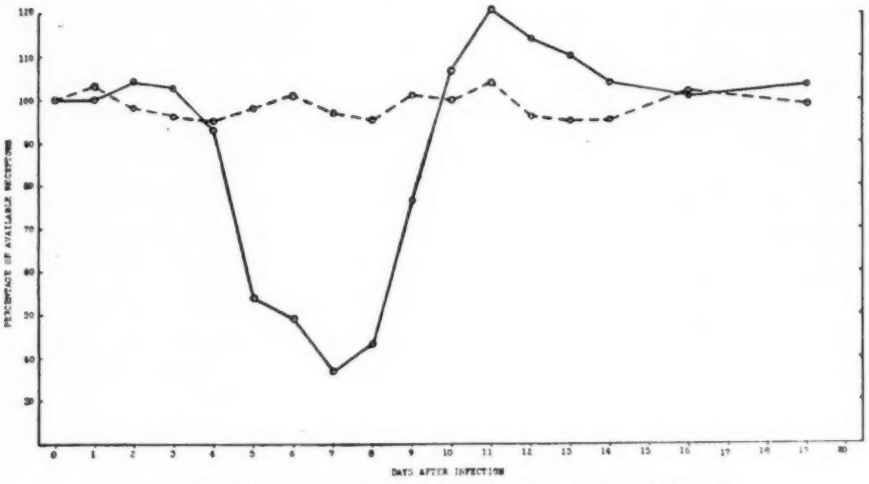

Fig. 1. Virus receptors in lungs during experimental influenza.

——— mice infected with 1 ID_{50} of MEL (A) virus.
– – – – normal control mice.

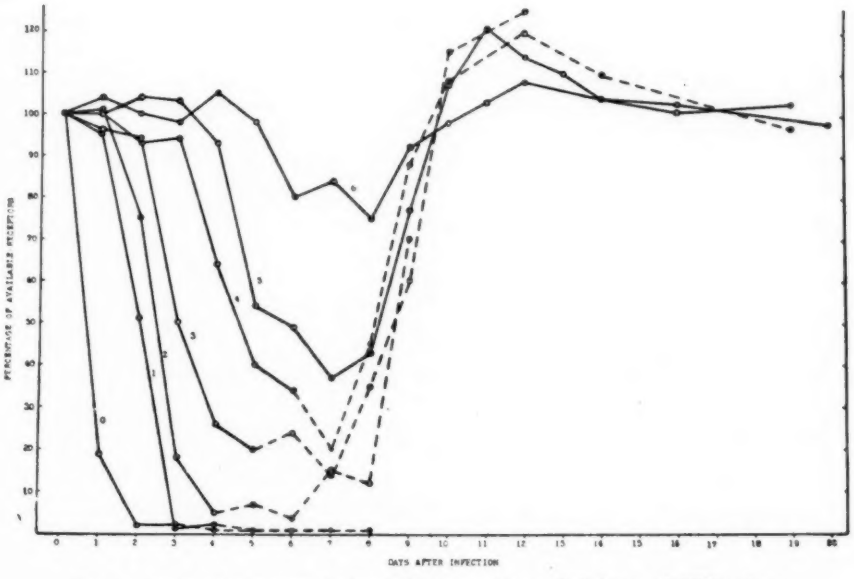

Fig. 2. State of receptors during infection with graded doses of MEL virus.

The numbers on the curves give the dilution factors of the infective inoculum in log_{10} units; "5" equals 1 ID_{50}. Where more than $\frac{1}{3}$ of the animals died spontaneously, the reactions of the survivors are indicated by broken lines.

tors is more complete. The period of hypercompensation does not last long, and from the fourteenth day onwards the number of receptors does not differ from that of normal animals.

Variations in the amount of virus contained in the infective inoculum cause readily demonstrable quantitative changes in the sequence of destruction and regeneration of receptors. However, the type of response is qualitatively identical in each case. Fig. 2 gives the results of an experiment in which groups of mice were infected with dilutions of Melbourne virus ranging from 1:1 to 1:1,000,000. Since many of the mice receiving heavier inocula died between the 4th and 9th days of the experiment, the regeneration part of the curves (in broken lines) does not represent homogenous populations but the averages of the mice surviving experimental infections.

There appears to be a positive correlation between the virus content of the infective inoculum and the degree to which receptors are destroyed. After heavy inocula the number of receptors begins to fall shortly after infection and stays at a minimum level for a considerable time. Similarly, the rate and extent of hyper-regeneration, and the time for which the number of receptors is above the normal, exceeds values found after infections with dilute inocula.

The relation of these phenomena to the virus content of the lung tissue and the respiratory tract will be shown below.

The State of Receptors after Vaccination.

To determine whether the number of receptors showed any change after vaccination or the administration of live virus by routes other than the respiratory one, the following experiments were performed.

Groups of mice were given intranasal inocula of 0·05 ml. allantoic fluid containing killed Melbourne virus. Two types of vaccine were used: "formol vaccine" prepared by the addition of 0·05 p.c. formaldehyde (i.e. 0·125 p.c. formol) to the allantoic fluid which was kept subsequently at 37° C. for 12 hours, and "heated vaccine" prepared by heating allantoic fluids for 60 minutes at 56° C. The infectivity of virus is destroyed by both treatments; the enzymic activity of the heated vaccine is significantly reduced (by more than 99 p.c.), while the formol vaccine retains it to the full. The antigenicity (capacity to bind specific antibody *in vitro*) is practically unaltered in either case.

0·10 ml. amounts of the same vaccine, or a similar volume of live virus were given to other groups of mice intraperitoneally. The living virus was included because statements have appeared in the literature that experimental animals have been infected by this route. If this were true, one would have to suppose that receptors distinct from the respiratory ones would be involved in the infective process, as the virus could approach the susceptible cells either through the lymph or blood stream or through the tissues but not via the mucosa of the respiratory tract, whose receptor content is determined by the technique used.

It does not seem necessary to give the results of these experiments in detail, as all were clearly negative. There was no change in the number of receptors either after intranasal or after intraperitoneal administration of killed (non-infective) virus. Similarly, the number of respiratory receptors stayed constant for the whole twenty days following the intraperitoneal injection of 1,000,000 ID_{50} of active virus. This latter finding was a surprising one in

view of reports in the literature, and would indicate that, contrary to expectations, mice cannot be infected by active virus which is not given by the respiratory route. Further experimental evidence bearing out the same point will be given in the third paper of this series, and the questions immediately arising will be discussed there.

The State of Respiratory Virus-Inhibitors after Infection.

In previous work (Fazekas de St.Groth, 1948 a) it has been observed that the first bronchial washings of normal mice invariably contained measurable amounts of an inhibitor for heated LEE virus. Regarded as an undesirable complication at that time, the inhibitor was removed by repeated washings previous to all experiments. The observation of Burnet (1948 a) that the respiratory mucus is an alternative substrate for the viral enzyme to that contained in cellular receptors, and that the presence of abundant mucoid secretion might prevent contact between the virus particle and the future host cell not only as a physical barrier but also as a competitive "receptor", renewed interest in the inhibitor.

Furthermore, as the respiratory mucoid inhibitor is destroyed *in vitro* by viruses of the influenza group, there seemed to be a possibility of developing a simple diagnostic test for the acute stages of influenza, namely by testing for the presence of inhibitor in respiratory secretions. It was expected that this would disappear whenever a sufficient amount of enzymically active virus was present in the respiratory tract.

Investigation of this point was included in the experiments already described on changes in the cell receptor level with infection. Immediately after excision the respiratory tract of all lungs was washed with three changes of 0·50 ml. normal saline, and the inhibitory titre of the fluids determined. The results shown in Table 1 give the total amount of removable inhibitor, i.e. the sum of the three titres, after the actual readings have been corrected for the "residual quantities". (For the method of computation see Fazekas de St.Groth, 1948 a.) For convenience of comparison with Fig. 1, the results with an ID_{50}[2] are plotted in Fig. 3.

In the group of mice infected with a 10^{-5} dilution of Melbourne virus the inhibitor content of bronchial washings dropped abruptly to less than 3 p.c. of its original value between the first and second day after infection. During the following three days there was no inhibitor present, but by the time the virus concentration of the washings began to decrease the inhibitor reappeared and there was a very marked overproduction from the seventh day on. This coincides with the time of regeneration (and hyper-regeneration) of receptors, but exceeds it in that the inhibitor content of the washing fluids, on the twelfth day for example, was approximately ten times higher than in normal lungs, and even on the twentieth day, at the termination of the experiments, it had not yet reverted to the pre-infection level. However, the tendency for such a reversal is indicated from the fourteenth day onwards.

In the experiment with graded infective doses of virus the differences are less marked than the differences in the behaviour of receptors in the same lungs. Table 1 gives the results and it can be seen that after intranasal administration of 1 to 1,000,000 infective doses of live

[2] ID_{50} (infective dose) is the amount of virus which causes an average 50 p.c. lung consolidation on the seventh day after intranasal instillation.

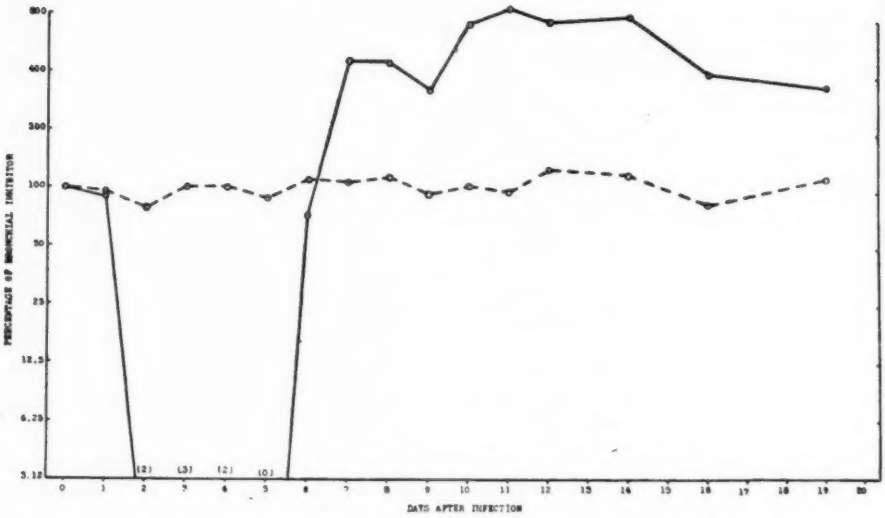

Fig. 3. Inhibitor content of bronchial washings in experimental influenza.
——— mice infected with 1 ID_{50} of MEL (A) virus.
- - - - normal control mice.

virus the respiratory inhibitor for heated LEE virus disappeared in the first 48 hours. Irrespective of the infective dose, the inhibitor reappeared roughly about the sixth day, and there was a marked hyperproduction for at least 14 days. In the second part of the Table, due to the death of several animals infected with large amounts of virus, the values given are not true averages in that they represent only the survivors of each experimental group. That the disappearance of respiratory inhibitor is a constant feature of the process of influenza infection in mice, and that during recovery from the attack large amounts of it are produced, are clearly seen from the results.

Respiratory Inhibitors after Vaccination.

As in the case of receptors, all experiments gave clear-cut negative results. During the twenty days following intranasal instillation of formolized or heat-killed influenza virus there was no significant change in the amount of inhibitor removable by bronchial washing. Similarly, after the intraperitoneal administration of these vaccines or of 10^6 infective doses of active virus, neither a decrease nor an increase was observed in the inhibitory titres.

The fact that intranasal formol vaccine did not cause any change is unexpected, as the amount given is capable of destroying in one hour a hundred times as much inhibitor *in vitro* as is present in the respiratory tract. The bearing of this finding on the virus-host cell relationship will be discussed below. The lack of change after active virus given peritoneally is consistent

TABLE 1.

Inhibitor content of bronchial washings in experimental influenza.

(The values are expressed as percentages of the average found in normal mice.)

Dilution of infective inoculum	Days after infection															
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	19	20
10^{0}	0	0	1 (98)	0 (62)	7 (35)	75 (27)	510 (12)	(2)	(0)							
10^{-1}	6	0	2	0 (90)	2 (54)	1 (21)	280 (14)	440 (6)	(0)							
10^{-2}	40	3	1 (97)	1 (77)	5 (38)	40 (14)	600 (14)	340 (8)	440 (6)							
10^{-3}	70	2	2	0	2 (94)	5 (53)	4 (25)	370 (18)	510 (11)	340 (8)		600 (6)				
10^{-4}	105	3	1	2	4	135 (84)	390 (54)	600 (50)		440 (50)		810 (48)	600 (48)		440 (48)	
10^{-5}	92	2	3	2	0	76	445 (99)	440 (97)	340 (97)	710 (96)	901 (96)	770 (96)	810 (96)	400 (96)	370 (96)	
10^{-6}	95	1	2	1	1	40	280	510	390	390	600	810	710	280		390

(The figures in brackets indicate the percentage of survivors, if less than 100.)

with the lack of change in the number of receptors: both findings support the view that the future host cell is susceptible to viral infection only from its respiratory surface.

The Virus Content of Lungs and Bronchial Washings after Infection.

As has been shown in recent years, the destruction of both cellular receptors (Hirst, 1942) and of inhibitors (Burnet and collaborators, 1947–49) has to be attributed directly to the infective particle. If there is a decrease in the number of receptors or the amount of inhibitor, an increase in the virus content is to be expected, *a priori*. This expectation has been fully verified by the experiment.

It is sufficient to give the data obtained with a 10^{-5} dilution of Melbourne virus as infective inoculum. This amount of virus was chosen because (a) it causes a typical disease in all mice with an average 50 p.c. lung consolidation on the seventh day; (b) more than 90 p.c. of the animals survive; (c) the changes in available receptors and inhibitors are not abrupt as with heavier inocula, and not indefinite as after smaller infective doses. It has to be added that all results obtained in this group hold qualitatively for all the groups tested (10^{0}–10^{-6}), and it is only for the sake of brevity and convenience that these are not reported in detail.

The haemagglutinin content of the first three bronchial washings was determined, and the sum of agglutinating doses recovered will be called "free virus". The amount of virus not removable by washing of the respiratory surface is denoted "virus in lungs". Each lung was ground with powdered alundum and emulsified in 1·0 ml. of normal saline. The supernatant fluid after centrifugation at low speed was tested for haemagglutinin. Inhibitor insensitive fowl cells were used in all titrations.

It is of interest that not all the virus can be liberated from lungs, even by prolonged grinding. If the ground lungs are treated with RDE, a further amount of haemagglutinin appears[3]. As the ratio between the masked and apparent virus in lungs seemed to be constant in our experiments, the values given in the Table refer always to the amount obtainable by simple grinding, i.e. apparent virus.

TABLE 2.

Haemagglutinin (virus) content of the respiratory organs in experimental influenza.

Mice infected with 0·05 ml. of a 10^{-5} dilution of Melbourne (A) virus.

Virus content of	Days after infection									
	1	2	3	4	5	6	7	8	9	10
bronchi	0	4	7	11	10	8	5	1	0	0
lung tissue	0	15	42	190	140	140	120	24	0	0

The values are the geometric mean titres of a group of ten animals.

Table 2 shows the haemagglutinin recovered as free virus from the bronchi and the amounts contained in the lung tissue after infection with Melbourne virus. After an incubation period of a day detectable quantities of virus appeared, simultaneously both in the supernate of ground lungs and in the bronchial washings. There was a steady increase during the first four days after which came a plateau at the maximum level. This was in turn followed

[3] This experiment was suggested by Mr. E. L. French, of this Institute.

by a rapid decrease in the virus content. After the eighth day there was no demonstrable virus in the infected mice. As will be seen in a later paper of this series, this time sharply coincides with the appearance of free, specific antibody in lungs and bronchial washings. (With heavier inocula the peak is reached earlier, the plateau is higher, but the decrease appears similarly between the sixth and eighth days.)

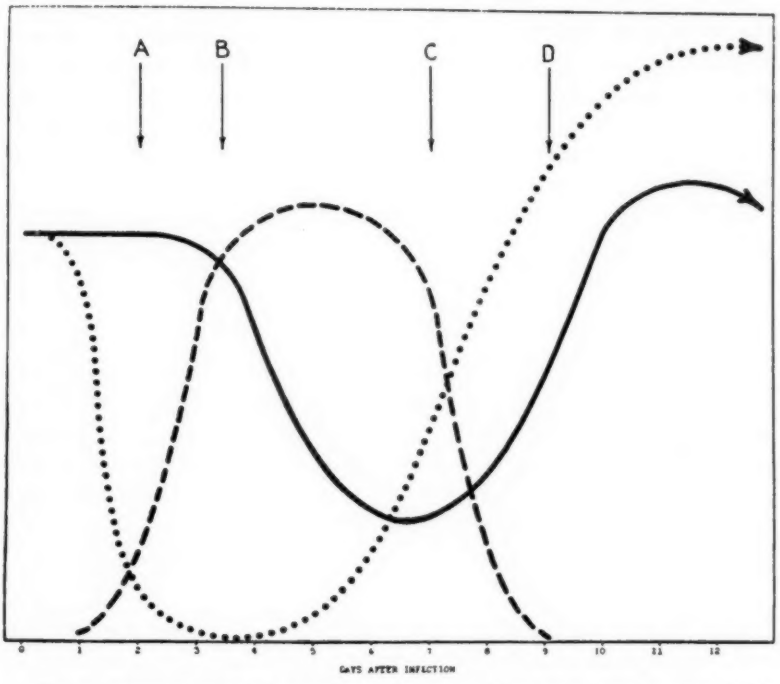

Fig. 4. Correlation of changes in receptors, inhibitor and virus content of the respiratory tract during experimental influenza.

(The smooth curves are constructed from the data of Figures 1 and 2 and Table 2.)

———— available receptors; . . . . . inhibitor; – – – – free virus.

A = appearance of virus—disappearance of inhibitor.
B = peak of virus content—progressive destruction of receptors.
C = decrease of virus—increase in inhibitor and receptors.
D = disappearance of virus—hyper-regeneration of receptors, over-production of inhibitor.

It is evident that the appearance of free virus in the respiratory tract is immediately followed by the disappearance of all inhibitor. The number of available receptors, however, is not changed till a critical level of free virus is reached. This occurs on the fourth day, and though there is no further increase of virus in the following four days, the number of receptors decreases steadily, as long as there is free virus present.

The decrease in virus content marks the turning point in the state of receptors and inhibitors. After the period of destruction regeneration starts and, as shown above, hypercompensates for the loss inflicted by the presence of active virus. That the process of regeneration is at work during the whole time of the experimental disease maintaining a dynamic equilibrium rather than starting only after virus has disappeared, is borne out by the fact that (a) the level of receptors is not reduced to zero, though they are in constant contact with amounts of active virus sufficient to destroy receptors in the excised lung in less than six hours, and that (b) the respiratory inhibitor reappears *before* the virus has disappeared completely, i.e. in the earliest stages of recovery there are large amounts of virus in the respiratory tract co-existing with inhibitor destructible by the virus.

Fig. 4 gives a graphical representation of the changes in receptors and inhibitors as correlated wtih the actual concentration of virus in the bronchi and the lung tissue of infected mice.

DISCUSSION.

In the present paper it was shown that intranasal administration of active virus to the mouse (experimental infection) is invariably followed by the disappearance of inhibitor from the respiratory tract, and by a decrease in the number of receptors on the lining cells. The rate and extent of this destruction is a function of the amount of free virus in the respiratory tract; the inhibitor appears to be far more susceptible than cellular receptors. This finding is impossible to explain on the results of *in vitro* experiments, in which active virus shows high preference for cellular receptors. However, it could be understood if the physiological activities of the susceptible cell are taken into account. As has been shown in previous work (Fazekas de St.Groth, 1948 c; Fazekas de St.Groth and Graham, 1949), living susceptible cells take up the adsorbed virus particle, irrespective of whether the receptor involved in the virus-host cell combination is destroyed during the process. Obviously, this is pragmatically equivalent to assuming that all the rest of the receptors, even on the same cell, remain capable of adsorbing virus when tested *in vitro*. A similar relationship is well recognized in bacterial cell-bacterial virus systems. Thus in a competition for virus between the cell and the respiratory inhibitor, the virus will combine with the cell, but will not destroy all of its receptors. Measurable receptor destruction will begin only when the number of susceptible cells (those capable of ingesting the adsorbed particle) is significantly reduced, and the virus in the respiratory tract is free to "browse" on the surface of the remaining cells, as it does on dead cells or inhibitors (Burnet, 1948 a; Anderson *et al.*, 1948). This limiting exhaustion of susceptible cells will occur at different times in different parts of the respiratory organs, as it is conditioned by

and will vary with the anatomy of the respective regions. Free virus is bound to appear and accumulate at points where the initial cell-lumen ratio was small (trachea, larger bronchi), and this free virus—demonstrable in the above experiments from the very early stages of infection onwards—is capable of destroying completely the inhibitor passing through that portion of the air passages.

If this hypothesis holds, it is to be expected that practically all of the inhibitor should disappear immediately after free virus has appeared in the respiratory tract; the number of receptors should fall noticeably only when there are no further susceptible cells, i.e. when the virus concentration has attained its maximum in the lungs. The experimental results (e.g. those summarized in Fig. 4) strongly favour this conception.

The continued presence of large amounts of infective virus stagnating in the upper regions of the respiratory tract would furnish a rational explanation for the finding of Straub (1940) and Taylor (1941) that intranasal instillation of some neutral fluid, e.g. normal saline, to animals in the early stages of influenza infection increased the extent of lung consolidation and the incidence of death due to experimental infection. The second inoculum necessarily passing through the trachea and larger bronchi, would wash down the virus accumulated there to parts of the respiratory tract which escaped primary infection with the relatively small infective doses after which the phenomenon was demonstrable. That a second inoculum itself does not alter the course of the infective process, is borne out by Taylor's finding that specific antiserum given in the same way did not cause any change in the final outcome of the disease.

Measurable regeneration of receptors and reappearance of inhibitor coincides with the decrease in free virus, which in turn is conditioned by the appearance of specific antibody. Both processes are typically hypercompensatory, the production of inhibitors more so than that of cellular receptors. There are distinct features which would indicate that both start earlier than is apparent from the experiments, as in the presence of virus, there is a dynamic equilibrium maintained between destruction and regeneration.

The main interest of these findings lies in their bearing on the host's susceptibility to influenza infection. From investigations restricted to the histology of the nasal mucosa of the ferret, Francis and Stuart-Harris concluded that experimental influenza is followed by a period of absolute resistance to reinfection (2–3 weeks). The morphological basis of this insusceptibility would be the different type of cells replacing the original epithelium (Francis and Stuart-Harris, 1938 a, b; Stuart-Harris and Francis, 1938). On the other hand, Sugg and Magill (1946, 1947) were able to show beyond doubt that previous infection with either type of influenza virus does not alter the host's susceptibility to the heterologous type. This latter finding is in accord with

the sparse data obtained in human epidemics. However, due mainly to the nature of the methods used, their observations were made in the later phases of recovery from previous infection. If the number of available receptors can be taken as an index of the virtual infectibility of cells, our results fully agree with Sugg and Magill's interpretation. In non-lethal infections, at any stage of the process, there are cells capable of adsorbing influenza virus in the respiratory tract. Only after experimental infections with very heavy inocula is there receptor destruction sufficient to make further adsorption of virus to cells impossible. This particular case, however, is of theoretical interest only, since all mice in which such extensive receptor destruction was shown succumbed to the disease within six days after infection.

No special effort was made to determine the eventual causal relationship between receptor destruction and the lethality of influenza infection, as it has been clearly shown (Stone, 1947 b, 1948 a, b; Fazekas de St.Groth, 1948 b) that the mere absence of receptors brought about by destruction by RDE, or their modification by periodate (Fazekas de St.Groth and Graham, 1949) does not affect the viability of the experimental animals used. The disappearance of receptors in the respiratory tract thus appears to be a symptom rather than a cause of the lethal outcome of influenza infection.

SUMMARY.

In mice experimentally infected with influenza virus the number of available virus-receptors in the respiratory tract drops during the acute phase of the disease. Measurable receptor destruction starts at the time when virus concentration in the lungs has attained its maximum; its extent is proportional to the size of the infective dose.

Coinciding with the appearance of specific antibody and the decrease in the virus content of the lungs, regeneration of receptors is demonstrable. Evidence is furnished that this regeneration starts earlier but is partly masked by the presence of enzymically active virus capable of maintaining a dynamic equilibrium between destruction and regeneration of receptors.

The mucoid inhibitor for heated LEE virus normally present in the respiratory tract, disappears within 48 hours after infection and does not reappear till the sixth day. During the early stages of recovery (8th to 20th day) the amount of inhibitor in bronchial washings is approximately ten times more than in normal mice.

Administration of large doses of killed virus vaccines (nasally, peritoneally or subcutaneously), or of live virus by routes other than the nasal, does not cause any change in the number of available receptors or the inhibitor content of the respiratory tract.

The mechanism of infection by and resistance to influenza viruses is discussed in the light of the findings and the conclusion is reached that the changes described are insufficient to cause any difference in the host's susceptibility to super- or re-infection.

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STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

II. PRODUCTION OF VIRAL ANTIGENS

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Investigations into the changes caused by influenza virus infection in experimental animals have been conducted by several workers in the past; their results answered a large portion of the questions posed. Resumption of research along similar lines, however, does not seem unjustified since previous studies were mostly restricted to circumscribed problems, and it is not easy to integrate the results and correlate data obtained by different workers using different methods and materials and different criteria of evaluation. Furthermore, due to the inherent inaccuracy of the techniques available at the time, most of the early findings are of a more or less qualitative character.

Changes in the level of receptors and inhibitors in the respiratory tract during the processes of infection and immunization have been described in the previous paper (Fazekas de St.Groth, 1950). The present study is concerned with the quantitative estimation of influenza antigens in mice receiving graded doses of live or inactivated virus by different routes.

In order to have a solid basis for correlating the numerous and simultaneously occurring biological reactions, a single, large group of animals was used in the experiments—the same on which the previous study was made, and whose later response will be described in the following two papers of this series. By keeping uncontrollable variables uniform for the whole series, this arrangement allows of more valid conclusions, both in the direct comparison of the processes concerned and in the study of their time relationships.

MATERIALS AND METHODS.

A full description of *materials* is found in the first paper of this series (Fazekas de St.Groth, 1950), and it is only the special techniques concerned with the vaccination of mice and estimation of antigens which are given here.

Titration of Virus Haemagglutinin.

In view of the small volume of the samples and the low starting dilutions, an alternative technique of titration was devised. This was used in addition to that given in Paper I. Extensive control experiments have shown that titres obtained by the two methods did not differ and had standard errors of the same magnitude ($\sim \pm 0{\cdot}40$ $\log_2$ unit).

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra, Australia.

Two-fold dilutions of the samples were made in 0·25 ml. volumes of 0·5 p.c. fowl cells, or if the sample was very small, in 0·125 ml. volumes of 0·5 p.c. cells; in the latter case tubes of 5 mm. internal diameter were used instead of 10 mm. Readings were made after 30 minutes' standing at room temperature, during which time the cells settled completely. Partial agglutination was taken as the end-point. The titres refer not to the final volume in the test but to *half of the final volume* in order to obtain results directly comparable with those of the standard test where titres refer to the volume before addition of an equal volume of 1 p.c. red cells. By this means the definition of one agglutinating dose (AD) is the same for both types of techniques: it is the amount of virus which causes partial agglutination of 0·25 ml. 1 p.c. fowl cells at room temperature.

The Complement Fixation Test (CF-test).

The technique of Donnelley (1950) was used; for details and discussion see the original paper.

The standard diluent, *Ca-Mg-saline*, was prepared according to Mayer *et al.* (1946); it contained 9·00 gm. NaCl, 0·575 gm. 5,5-diethylbarbituric acid, 0·375 gm. Na-5,5-diethylbarbiturate, 0·168 gm. $MgCl_2 \cdot 6H_2O$, 0·028 $CaCl_2$ in 1,000·0 gm. distilled water. The autoclaved, sterile solution had a pH of 7·25.

Specific *antisera* were obtained from ferrets convalescent after experimental infection with MEL (A) or LEE (B) viruses. On the day of the test the standard serum was diluted 1:5 and inactivated by heating for 15 minutes at 62° C.; it was used without further dilution. The *indicator system* consisted of 3 p.c. sheep cells sensitized with 4 units of rabbit amboceptor.

Complement (guinea-pig serum) was diluted to contain three 50 p.c. haemolytic doses in the standard volume.

Samples in which the presence of *antigen* was to be determined (ground lungs or bronchial washings) were absorbed with 10 p.c. human erythrocytes in order to remove substances interfering with the test.

Doubling dilutions of the samples were made by the drop-technique. Equal volumes (2 standard drops = 0·06 ml.) of the antigen dilution, complement and standard antiserum were mixed in that order, shaken and incubated for 60 minutes at 37° C. Then two drops of sensitized sheep cells were added, and the test read after a further 30 minutes' incubation at 37° C. Fifty p.c. lysis was taken as the end-point, the titres refer to the initial dilution of antigen (0·06 ml.), not to the final volume in the test (0·24 ml.). Each sample was tested for anticomplementary property under the conditions of the test, and in each set of titrations positive and negative serum and antigen controls were included.

Preparation of Vaccines.

The Melbourne (A) and LEE (B) strains of influenza virus were used in the experiments, in the form of infected allantoic fluids of high titre. Freshly harvested fluids are designated as *active or live virus*, showing 50 p.c. infectivity end-points in eggs when diluted 10^{-8}–10^{-9}. Two types of inactive vaccines were used: *formol vaccine* (active virus + 0·05 p.c. formaldehyde, incubated at 37° C. for 12 hours) and *heated vaccine* (active virus heated at 56° C. for 60 minutes). The latter two did not infect eggs at any dilution. As a rule the vaccines were used within four days after their preparation, in the meantime they were stored at 4° C.

Handling of Experimental Animals.

Mice were obtained from the Institute's breeding rooms at the age of 6 to 7 weeks, as a rule 60 p.c. females and 40 p.c. males. Sexes and ages were uniformly distributed among the experimental groups. Ten animals were housed together and given their usual full diet. No intercurrent disease was observed during the period of our study, and the only non-specific deaths (0·3 p.c.) were due to bronchopneumonia following the nasal instillation of fluid.

Three methods of vaccination were used. *Intranasal instillation* was done under light aether-chloroform (2:1) anaesthesia, 0·05 ml. volumes were given. *Intraperitoneal* injections of 0·10 ml. were made in the right inguinal region, and *subcutaneous inoculations* (0·10 ml.) into the loose connective tissue of the lumbar region. The last two were performed without anaesthesia.

Technique of Experiments.

The work reported in the first five papers of this series followed a uniform technical plan. The different tests were co-ordinated in the following way. Large groups of mice (1,000–1,400) were used in each experiment, inoculations (nasal, peritoneal or subcutaneous) being done always on the same day, the time interval between the first and last animal never exceeding three hours. On each of the following days ten animals of each group were killed by over-anaesthetizing. Their blood was collected from the severed art. subclavia. The lungs were excised by the technique previously described, and the respiratory surface washed with 0·5 ml. normal saline. (A group of three experienced workers prepares on the average 60–80 lungs per hour.) Each *bronchial washing*—immediately after having been collected—was divided into two lots, one for the estimation of haemagglutinin, the other for the titration of inhibitor and complement-fixing antigen. The latter sample was absorbed with 10 p.c. packed human red cells in order to remove the virus from it. Then the lungs were removed from the cannulae, the mediastinal structures (trachea, large vessels, oesophagus, thymus, etc.) removed and the remainder ground with sterile alundum and suspended in 1·0 ml. saline. Half of this volume was used for haemagglutinin titrations, the other half—similarly to the bronchial washings—was absorbed with 10 p.c. human erythrocytes and thus prepared for the CF-test.

Individual samples were always tested separately; all haemagglutinin titrations were performed within 6 hours after killing the animals, the complement fixation tests within 30 hours. The samples were stored at 4° C. between the tests, no preservative was used.

In groups where mice were expected to die from experimental infection a larger number of animals was inoculated to allow for this loss.

EXPERIMENTS.

Research in the last few years has shown that the virus particle itself is the agent concerned in the haemagglutination reaction of influenza virus-containing fluids and organ extracts. Hence, the haemagglutinin titre of any sample would be a satisfactory measure of its content in free virus, provided the material contained no other agglutinin against the cells used in the test, and no substance capable of inhibiting the viral agglutinin, i.e. masking the reaction.

Since the main experiments of this study aimed at determining the amount of influenza virus in the lung tissue and in the respiratory passages of mice under various conditions, in preliminary titrations normal and infected lungs were tested to determine whether they contained any substance which would either (a) agglutinate fowl red cells or (b) inhibit the haemagglutinating activity of influenza viruses used in this study.

"Normal lungs" were obtained by grinding the excised lungs of 10 healthy six-weeks-old mice in 10 ml. normal saline with sterile alundum, and removing the supernatant fluid after centrifuging at low speed. *"Infected lungs"* are those of mice given an intranasal inoculum of virus-containing allantoic fluid four days before; a pool of 10 lungs showing 25–75 p.c.

consolidation at the time of excision was ground and centrifuged as described above. The samples were diluted 1:5 in normal saline before being used in the test. *Bronchial washings* (''normal'' and ''infected'') were taken from the respective lungs before grinding by the technique described under Methods. Undiluted fluids were tested.

The *virus* (infected allantoic fluid) used in this experiment was diluted to give haemagglutinin titres of 80 by the standard technique. The specific *antisera* were made up in normal saline to contain in the standard volume antibody sufficient to neutralize ten times the amount of virus used in the tests.

TABLE 1A.

The effect of ground mouse lungs on the haemagglutination test.

Sample	Fowl cells											
	Lipid sensitive						Lipid insensitive					
	Inhib. sens.			Inhib. insens.			Inhib. sens.			Inhib. insens.		
	1	2	3	1	2	3	1	2	3	1	2	3
Normal lungs	+	+	+	+	+	+	—	—	—	—	—	—
Normal lungs + A-virus	+	+	+	+	+	+	+	+	+	+	+	+
B-infected lungs	+	+	+	+	+	+	+	+	+	+	+	+
B-infected lungs + anti-B serum	+	—	±	—	±	—	—	—	—	—	—	—
B-infected lungs + anti-B serum + A-virus	+	+	+	+	+	+	+	+	+	+	+	+

A-virus = Melbourne (A) strain of influenza virus.
B-virus = LEE (B) strain of influenza virus.
Antisera = Sera of ferrets convalescent from experimental infection.
Lungs = a pool from ten mice made up in 50 ml. saline.
+, ±, —, = complete, partial and no agglutination.

TABLE 1B.

The effect of bronchial washings of mice on the haemagglutination test.

Sample	Fowl cells											
	Lipid sensitive						Lipid insensitive					
	Inhib. sens.			Inhib. insens.			Inhib. sens.			Inhib. insens.		
	1	2	3	1	2	3	1	2	3	1	2	3
Normal washings	+	+	+	±	±	+	—	—	—	—	—	—
Normal washings + A-virus	+	+	+	+	+	+	+	+	+	+	+	+
B-infected washings	+	+	+	+	+	+	+	+	+	+	+	+
B-infected washings + anti-B serum	±	—	—	—	±	—	—	—	—	—	—	—
B-infected washings + anti-B serum + A-virus	+	+	+	+	+	+	+	+	+	+	+	+

Washings were made on excised lungs with 0·50 ml. of saline.

Fowl cells, in relation to their behaviour towards viral agglutinins, can be grouped according to two principles. They are either sensitive or insensitive to lipid agglutinins (Stone, 1946), and vary on a continuous scale in their suitability as detectors of the haemagglutination-inhibition by mucoids. For the sake of simplicity only the extremes will represent this

latter group, and will be called "inhibitor sensitive" or "inhibitor insensitive" cells. In the actual experiment three fowls from each of the possible four combinations were chosen. The reactions of their cells are tabulated below.

0·25 ml. volumes of the ingredients were mixed, incubated for 5 minutes at room temperature and then a 0·25 ml. sample tested against an equal volume of 1 p.c. fowl cells. Tables 1A and 1B give the result of this qualitative test.

The results show that both ground lungs and bronchial washings of normal or infected mice contain a lipid-agglutinin, but no other substance (with the exception of virus in infected lungs) which would agglutinate lipid-insensitive fowl cells. Haemagglutination by live influenzà viruses is not inhibited to a significant degree by either lungs or respiratory secretions, therefore the use of inhibitor insensitive cells in virus titrations is not essential. The findings bear out that live virus can be estimated satisfactorily by the haemagglutination reaction in infected lungs or bronchial washings, provided lipid-insensitive cells (Stone, 1946) are used in the titrations.

Dilutions of recently harvested allantoic fluids of high haemagglutinin titre were made up in horse serum saline, ranging from 10^0 to 10^{-10}. 0·05 ml. volumes of these dilutions were instilled intranasally under light aether-chloroform (2:1) anaesthesia to large groups of six-weeks-old mice. The heavier inocula of live virus caused typical disease in all animals. Irrespective of the number of spontaneous deaths, ten mice of each group were killed on each of the fourteen days following instillation. Bronchial washings were taken from each mouse and the lungs ground as described under Methods. All samples were titrated for haemagglutinin and complement-fixing antigen; values given in the Tables below are the geometric mean titres of a group of ten surviving animals on the day indicated.

The Virus Content of Lungs after Experimental Infection and Vaccination.

Both types of influenza virus were used in the experiments, the results showing but minor quantitative differences; the findings with the Melbourne strain of influenza A are given in detail.

TABLE 2.

Haemagglutinin titre of experimentally infected mouse lungs.
Virus strain Melbourne: (A)

Dilution of inoculum	Days after inoculation									
	1	2	3	4	5	6	7	8	9	10
10^0	160	184	145	42	14	3	0	0		
10^{-1}	34	100	243	76	16	12	0			
10^{-2}	9	172	304	241	114	86	16	0	0	
10^{-3}	0	52	176	218	148	104	27	0	0	0
10^{-4}	0	64	112	164	182	120	34	0		0
10^{-5}	0	15	42	190	140	140	120	24	0	0
10^{-6}	0	7	30	82	110	26	12	0	0	0
10^{-7}	0	0	12	37	58	21	8	0	0	0
10^{-8}	0	0	0	0	3	7	0	0		0
10^{-9}	0	0	0	0	0	0	0	0		0

The values are the geometric mean titres of a group of ten animals.

It can be seen that multiplication of virus detectable by the haemagglutination reaction occurs in all groups up to 10^{-8} dilution of a virus preparation which causes 50 p.c. lung consolidation when given in a 10^{-5} dilution. The amount of haemagglutinin produced after infection with heavy or light inocula does not show very marked variations—a millionfold difference in the infective dose is only reflected by a less than threefold difference in the maximal virus titre of lungs. The daily averages, as shown in the Table, give the impression of more regularity than was actually observed. Individual titres, within each experimental group, were widely scattered and the extreme values differed—not infrequently—by more than fourfold. Though the height of the group-averages shows a slight positive correlation with the amount of virus given in the infective inoculum, the standard deviations of the mean values overlap extensively. It is not justifiable therefore, to attach much significance to these differences, and it can be concluded from the findings that multiplication of influenza virus haemagglutinin in mouse lungs is roughly uniform over the range of infections with 0·1 to 100,000 ID_{50}. After nasal inoculation of smaller doses the quantity of virus produced drops abruptly, and there is no detectable haemagglutinin in lungs of mice given a 10^{-9} dilution (0·001 ID_{50}) of virus.

The differences in the time curves of virus multiplication are more characteristic. Though, due to the marked individual variations, the data do not lend themselves readily to quantitative evaluation, the relationship existing between the size of the infective dose and the time interval after which the maximum of virus concentration in the lungs is reached cannot be overlooked. After heavy inocula the peak is reached within 24 hours (group 10^{0}), after a small dose only round the 4–5th day (groups 10^{-5}–10^{-7}). The ascending parts of the virus multiplication curves are much flatter than those obtained in work on allantoically-infected eggs. Judged by this criterion, the process of infection and multiplication in the two types of host cells seems to be at least quantitatively different.

Whatever its time of appearance or maximal titre in the lungs be, all virus detectable by the haemagglutinin test disappears between days 7 and 9. As will be shown in the following paper (Fazekas de St.Groth and Donnelley, 1950) this time sharply coincided with the appearance of specific antibody in the circulation and in the respiratory tract. In groups where virus concentration attains its maximum early, the decrease and disappearance sets in earlier. This statement holds for the range of inocula after which uniformly high haemagglutinin titres are reached in the lungs (10^{0}–10^{-5}). A discussion of these results follows later in the paper.

In the *second series of experiments* live virus was given by the peritoneal or subcutaneous routes. 0·10 ml. volumes of undiluted allantoic fluid of high haemagglutinin titre were injected. The lungs of ten mice were prepared daily after immunization, and tested for their

virus content. It does not seem necessary to give the results in detail, since all were clearly negative.

In the *third series of experiments* killed virus vaccines (inactivated by formaldehyde or heat) were administered by the nasal, peritoneal or subcutaneous routes. Again, there was no detectable haemagglutinin produced in the lungs during the 14 days after inoculation. As shown in the following papers, these vaccines were highly antigenic and the serological response was comparable to that observed after experimental infection.

These results bear out that influenza infection (i.e. multiplication of the virus in a susceptible tissue) follows *only* nasal instillation of live virus. Active virus given by extrarespiratory routes is as incapable of causing infection as inactivated vaccines (killed by formaldehyde or heat) given by any route.

The Virus Content of Bronchial Washings.

Bronchial washings from all mice used in the above experiments were tested. The data obtained with Melbourne (A) virus are given in detail. Table 3 refers to the same group of animals as Table 2, the two sets of results are directly comparable.

TABLE 3.

Haemagglutinin titre of bronchial washings from experimentally infected mice.
Virus strain: Melbourne (A).

Dilution of inoculum	Days after inoculation									
	1	2	3	4	5	6	7	8	9	10
10^{0}	28	34	30	12	16	8	0	0		
10^{-1}	4	26	38	34	28	16	4	0		
10^{-2}	0	30	38	42	32	28	8	0	0	
10^{-3}	0	16	32	28	28	16	18	6	0	0
10^{-4}	0	7	10	18	24	20	8	0		0
10^{-5}	0	4	7	11	10	8	5	1	0	0
10^{-6}	0	2	6	5	8	5	1	0	0	0
10^{-7}	0	1	2	0	2	0	1	0	0	0
10^{-8}	0	0	0	0	0	0	0	0		0
10^{-9}	0	0	0	0	0	0	0	0		0

The values are the geometric mean titres of a group of ten animals.

Again, as was observed in the case of virus in infected lungs, there is no significant difference in regard to the absolute amount of virus free in the respiratory tract between mice infected with 1 or 100,000 ID_{50}. The uniform, maximal response ends one dilution earlier (at 10^{-5}), and the last group showing detectable haemagglutinin in bronchial washings is 10^{-7}, while in lungs the limiting dilution was 10^{-8}.

Large quantities of virus are liberated into the air passages within 48 hours after infection with as little as 0·01 ID_{50}. The virus content stays at a fairly constant level in the bronchi till the fifth day; definite peaks are not

seen. Consequently, in the time curves there is not much more than a hint at a relationship with the infective dose, similar to that found in the corresponding lung tissue. These results would point at a dynamic equilibrium as the mechanism governing the concentration of virus in the respiratory tract, with a continuous liberation of fresh virus from the lining cells on one side and a continuous removal via the upper air passages, maintaining a constant level.

The amount of virus in the bronchi is roughly 1/5 of that detectable in the lungs after simple grinding. This ratio seems to increase near the time of disappearance of the virus, round the 6–8th day. However, it was not decided whether this was a genuine phenomenon or was caused only by the nature of the techniques used. The fact that the bronchial washings are taken by a gentle method, viz. simple rinsing of the respiratory surface, ensures the reliability of the obtained data. This cannot be said about the handling of lungs, since grinding disrupts the histological structure and is liable to bring together components (e.g. in the later stages virus and antibody) naturally separated from each other; thus the results show always the net reactions of the whole lung and not only those of the infected or infectible cells.

Negative results were obtained in the *second and third experiments.* There was no virus detectable by the haemagglutination reaction in the bronchial washings of mice which received 100,000 ID_{50} of live virus peritoneally or subcutaneously, or equivalent amounts of formolized or heated vaccines administered by the nasal, peritoneal or subcutaneous routes.

The "Complement Fixing Antigen".

The "small antigen" of influenza infected tissues has not been studied as extensively in the past as the virus particle itself. The lack of knowledge is best demonstrated by the fact that three contradictory hypotheses exist regarding the nature of the antigen, all based on the same available evidence, all equally acceptable and unconvincing. According to these the "S30" component of infected tissues might be either a precursor of the virus, or its breakdown product, or an abnormal metabolite of the cell unrelated to the virus but caused by its presence (and/or multiplication) in a living protoplasm. Investigations into the physico-chemical properties of the antigen furnished a number of data, none of which proved to be really decisive. Our study was confined to the quantitive estimation by serological methods of complement-fixing antigen in the animal organism. It was hoped that the correlation of its appearance, presence and disappearance with other reactions occurring in infected or vaccinated animals might shed some light on its nature and give a lead regarding its relationship with either the virus particle or the host cell.

Daily titrations of the CF-antigen content of lungs from individual mice were made on the same group on which the multiplication of virus was being followed. Uniformity of the technique was checked by the inclusion in each set of titrations of at least eight samples tested on previous days. Experiments devised specially to assess the reproducibility of results (Donnelley, 1950) show that the antigens and standard sera keep without any deterioration over the time of our study, and that the accuracy of the method is actually higher than that of a haemagglutinin titration. All titres less than 7 are regarded as non-specific, since our stand-

ard ferret antisera fixed complement in the presence of normal mouse lungs to titres between 2 and 7 (Donnelley, 1950). The results obtained after intranasal instillation of live Melbourne (A) virus are presented in Table 4.

TABLE 4.

Complement fixing antigen titre of experimentally infected mouse lungs.
Virus strain: Melbourne (A).

Dilution of inoculum	Days after inoculation									
	1	2	3	4	5	6	7	8	9	10
10^{0}	28	26	34	19	13	—	—	—		
10^{-1}	25	42	38	42	31	14	—			
10^{-2}	23	38	41	48	40	11	9	7	—	
10^{-3}	11	35	53	45	48	27	—	—	—	—
10^{-4}	—	24	40	42	40	21	9	—		—
10^{-5}	7	19	27	35	38	23	9	—	—	—
10^{-6}	—	12	19	24	21	14	—	7	—	—
10^{-7}	—	7	14	17	16	14	7	7	—	—
10^{-8}	—	—	9	12	12	7	9	—		—
10^{-9}	—	—	—	—	—	—	—	—		—

— = titre less than 7, considered non-specific reaction.
The values are the geometric mean titres from a group of ten animals.

The presence of influenzal complement-fixing antigen was demonstrable in all groups of mice in which virus could be detected by the haemagglutination test (10^{0}–10^{8}). Uniformly high titres were obtained in groups 10^{0}–10^{-5}, i.e. after infection with 100,000 or 1 ID_{50} of influenza virus. In groups given smaller inocula the amount of antigen produced was significantly less, and in group 10^{-9} (0·001 ID_{50}) there was no fixation outside the zone of non-specific reactions.

In contrast to the virus in the same lungs, the CF-antigen reaches its maximum level earlier and stays at this height for a considerable time. Variations within the individual groups are slight as compared with the marked scattering of virus titres; this regularity is well expressed in the smooth shape of the time-curves. The narrower range of error in averaging allows of stressing a point already suggestive in the virus titrations but which could not have been shown convincingly on the grounds of those results. After the *heaviest* inoculum of virus (10^{0}) the CF-antigen produced is significantly *less* ($P < 0{\cdot}03$) than after smaller infective doses. A similar paradoxical reaction is indicated in the haemagglutinin content of infected lungs (see Table 2), and also in the developing lungs lesions. The phenomenon will be discussed below.

The CF-antigen level drops from the fifth day onwards, and after the eighth day there are no titres above the range of non-specific reactions. This time coincides with the appearance of specific CF-antibody in the circulation (Fazekas de St.Groth and Donnelley, 1950). The sensitivity of the titrations was less in the last few days since the amount of complement used had to be increased in view of the marked anticomplementary property of infected lungs after the fifth day—a fact which can be attributed to the presence of an antigen-antibody complex (Donnelley, 1950).

Comparison of the individual titres of haemagglutinin and complement-fixing antigen, or even a comparison of the group averages, shows that the

ratio of the titres is not constant, that is, the amount of the two antigens varies independently. A similar lack of correlation was found also for the time relations of the two processes.

In two *further series of experiments* negative results were obtained: no CF-antigen was detectable in the lungs after peritoneal or subcutaneous injection of a million ID_{50} of live virus, or after the administration by any route of killed (inactivated by formaldehyde or heat) vaccines of comparable haemagglutinin content.

Complement fixation tests on *bronchial washings* showed no trace of antigen, even after experimental infection (nasal instillation of live virus).

These two sets of negative findings indicate that complement-fixing antigen is produced only in a susceptible tissue in which the virus multiplies, and that unlike the infective particle itself, the CF-antigen is not released from the cells, at least not—as in the case of virus—in quantities which would be detectable by our tests.

DISCUSSION.

A study of the antigens associated with influenza virus infection, viz. the virus particle itself and the smaller "soluble" antigen detectable by the complement-fixation test (called CF-antigen in our papers), shows that the appearance of both in the lungs of mice follows only nasal instillation of live virus, and not its administration by extrarespiratory routes (peritoneal or subcutaneous). Neither of the antigens is demonstrable after killed virus vaccines given by any route.

Though the virus and the CF-antigen coexist in infected lungs, there are several points of difference in their life-history. The CF-antigen appears almost concomitantly with the haemagglutinin, but reaches 50 p.c. of its maximum level approximately one day sooner than the virus. This fact militates against one of the possible hypotheses concerning the origin of the CF-antigen: it can hardly be the breakdown product of the virus, whose appearance it precedes. However, the experimental evidence presented in this paper is not sufficient to decide between the alternatives.

Whatever its origin be, the CF-antigen is detectable in all lungs in which virus grew sufficiently to react in the haemagglutination-reaction. Both are a sensitive measure of infection, giving positive results after as little as 0·001 ID_{50} or 0·0002 LD_{50}, as determined in the standard infectivity test.

As shown by Taylor (1941), after highly infective inocula the final virus concentration of the lungs is fairly uniform, the titres falling off only after infections with fractions of a minimum lethal dose. Our findings, obtained by a different technique, fully confirm his results and demonstrate the same phenomenon in the behaviour of the CF-antigen which also shows uniformly high final levels in lungs infected with 1 or 10,000 ID_{50}. This conclusion is

at variance with that of Eaton and Nicewonger (1940), though the two sets of experimental results are practically identical. The American authors claim a positive correlation between the size of the infective inoculum and the amount of CF-antigen produced. Their published data, however, seem to prove the contrary: after infection with 10^{-3} to 10^{-6} dilutions of PR8-mouse passage-virus (results with higher concentrations are not presented) the difference in the average CF-antigen titres between the second and sixth day are less than $\pm$ 0·6 $\log_2$ units, a finding identical with ours.

From the experimental data one can visualize the process of infection as spreading from cell to cell through their mucosal surface (for evidence see Paper I). The number of infectible cells in the lungs varies only within narrow limits. Supporting this view is the finding of high constancy in the number of virus receptors in normal lungs (Fazekas de St.Groth, 1948 a, b) and the strict positive correlation between the number of available receptors and infectibility (Stone, 1948). The fact that the maximum level of CF-antigen is so uniform in infected lungs ($<$0·5 $\log_2$ variation) is suggestive of the same point. In infections with one ID_{50} or more (1 to 100,000 ID_{50} in our experiments) presumably all infectible cells are infected: the uniform height of both the final virus and CF-antigen levels speaks for that. Reaching the maximum is a matter of hours after very heavy inocula (10^0 in the above experiments) or a matter of days after smaller ones (e.g. $10^{-5} = 1$ ID_{50}). The time elapsing between infection and the peak of virus concentration in the lungs is inversely related to the size of the inoculum, that is, a small number of virus particles takes a longer time to multiply sufficiently to infect all available host cells.

With very small inocula (less than 0·1 ID_{50}) the final antigen level is less than maximal. This is interpreted as the infection of only a proportion of the infectible cells, an assumption justified by the findings of Taylor (1941) on the enhancement of sub-lethal infections by washing down the virus into the lower parts of the respiratory tract, and by the results obtained in the study of virus receptors during the process of infection (Fazekas de St.Groth, 1950). It appears that some cells escape infection not because of the lack of free infective virus in the respiratory tract[2], but because of the upward carriage and elimination of the particles due to ciliary movement. Supporting evidence for this view was presented in Paper I; the constant haemagglutinin titres of bronchial washings also suggest a dynamic equilibrium maintained by continuous liberation of virus from infected cells and continuous excretion via the upper air passages. A small inoculum of virus will be adsorbed to the receptors it comes into contact with first, and the liberated newly-formed par-

[2] Even after inoculation of limiting doses, there is enough virus present in the bronchi to cause haemagglutination, i.e. more than 1,000 mouse ID_{50}!

ticles will follow a course determined by ciliary movement and will be incapable of causing centrifugal spread, i.e. of infecting areas not affected by the initial inoculum. Any method reversing this natural mechanism (e.g. Taylor's second inoculation) will infect further cells and bring about consequences normally following heavier initial inocula.

Whatever their final level, both virus and CF-antigen disappear at the time when the two types of antibody appear. This is clearly seen from the results when compared with those of the next paper of this series. Early appearance of specific antibody can explain the paradoxical reaction observed in both the production of the two types of antigen and the appearance of lesions. After very heavy inocula of live virus (more than 10,000 ID_{50}) the amount of antigens produced and the size of lesions is less than after smaller inocula (e.g. 1,000 ID_{50}). A similar relationship was recently described in two masterly papers of Schlesinger (1949 a, b), and his explanation seems to fit our results also. Very large amounts of virus are more than enough to infect susceptible cells, and the excess might (a) act as antigen and thus result in earlier and quantitatively increased antibody production, or (b) cause a "depression effect" demonstrated by Delbrück (1945) in the case of bacteriophage and by Henle and Rosenberg (1949) for influenza virus in the allantoic cavity of eggs. Further analysis of the phenomenon will be included in a later paper of this series. That the amount of antigen in the lungs is decisive for the shape of the time curve is shown by the earlier disappearance of virus from lungs where the maximum concentration was attained earlier, or translated into immunological terms, larger doses of antigen elicit an earlier and more pronounced antibody response (see Fazekas de St.Groth and Donnelley, 1950).

Of the two types of antigen only the virus particle appears in detectable quantity in the bronchial washings. The apparent ratio of virus in the bronchi to virus in the lungs is approximately 1:5 during the process of infection. This, however, is clearly not the true distribution, since it has been shown in the previous paper that, by the application of *V. cholerae* filtrates containing the "receptor destroying enzyme" (RDE), further large amounts of haemagglutinin can be liberated from infected lung tissue but not from bronchial washings; this would alter the ratio to 1:40. And even this might not be the final value; if lungs contained a substance similar to that found in chorioallantoic membranes by Isaacs and Edney (1950) which in the living cell is capable of firmly combining with the virus *in vitro* and masking it in the haemagglutination but not in the infectivity test, the true ratio might be even less than 1:40. While not being able to answer this question in a precise fashion, our experiments show that even with minimal infective doses (0·01 ID_{50}) large quantities of virus are present in the respiratory tract since the minimal

amount of virus causing haemagglutination contains approximately a thousand mouse infective doses. Further, after infection with 100,000 or 0·01 ID_{50} the difference in the virus content of bronchial washings is only approximately tenfold; this allows of the conclusion—at least in the case of mice—that irrespective of whether the animals were given inocula which cause fatal, clinical or subclinical infection, the amount of virus free and presumably excretable in their respiratory passages is not widely different.

SUMMARY.

The amount of virus haemagglutinin and of the soluble complement-fixing antigen (CF-antigen) was estimated in the lung tissue and the bronchial washings of mice given live and inactivated preparations of influenza virus by the nasal, peritoneal or subcutaneous routes.

After nasal instillation of live virus the maximal haemagglutinin titres are fairly uniform over the range of infections with 100,000 to 1 ID_{50}. After infections with fractions of an ID_{50} the titres reached are proportionately lower; no haemagglutinin was detectable after instillation of 0·0001 ID_{50}.

After inoculation of more than 1 ID_{50} the time between infection and the peak of virus concentration is inversely proportional to the size of the infective dose.

In all infections with 0·01 to 100,000 ID_{50}, virus detectable by the haemagglutination test is present in the lower air passages within 48 hours after inoculation.

Haemagglutinin disappears from both the lung tissue and the bronchi between the sixth to eighth days after infection, at the time when specific antibody appears.

Complement-fixing antigen was demonstrable in all lungs which contained haemagglutinin (after 100,000 to 0·001 ID_{50}); uniformly high titres were reached after infective doses higher than 1 ID_{50}.

The maximum CF-antigen concentration is attained somewhat earlier than that of the virus haemagglutinin.

CF-antigen does not appear in detectable quantities in the bronchial washings, and it disappears from the lungs at the same time as the haemagglutinin (6–8th day).

Administration of live virus by routes other than the nasal, or of killed vaccines by any route, does not give rise to haemagglutinin or CF-antigen production in the lungs.

The results are correlated, and the mechanism of infection, together with the factors determining its spread, are discussed.

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STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

III. THE ANTIBODY RESPONSE

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As with several antigen-antibody systems, it has been demonstrated that the serological response to influenza virus is proportionate to the logarithm of the immunizing dose. The further finding that epidemic or induced influenza infection was more commonly associated with low than with high antibody titres, encouraged workers to consider the easily measurable serum antibody level as a measure of immunity, this notwithstanding numerous and well recognized discrepancies.

Whatever its rôle be, antibody circulating in the vascular system is not directly involved in protection, since the virus, an obligatory respiratory pathogen, approaches and selectively attacks the susceptible cell from its mucosal surface without previously entering the blood stream. Considering the pathogenesis of the disease, a study of immunological mechanisms available at the portal of entry appeared more promising. It has been shown by Burnet, Lush and Jackson (1939) that nasal washings contained a substance capable of neutralizing the infectivity of influenza virus. The identification of this substance with specific antiviral immunebody is due to Francis (1940). In a series of studies (Francis, 1941 a, b; Francis and Brightman, 1941; Francis, Pearson, Sullivan and Brown, 1943) the immunological significance of antibody contained in nasal secretions was demonstrated, and Francis (1943) concluded—by inference—that this is an important, if not the most important determinant of specific resistance to influenza. Yet, the mechanism by which antibody reaches the respiratory surface has not been studied, nor have the factors governing its distribution between serum and bronchi. Consequently, the mechanism of immunity is imperfectly understood, and the persistent searching for better vaccines is a tribute to human optimism rather than intelligence.

The present study is confined to the systematic investigation of the antibody response in mice receiving graded doses of influenza virus, infective and inactivated, by different routes. All techniques used were purely serological, as are the conclusions. The findings in regard to active immunity and the significance of their correlation with the serological data, are the subject of the next paper of this series (Fazekas de St.Groth and Donnelley, 1950 b).

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MATERIALS AND METHODS.

Most materials and some of the methods were described in the first two papers of this series (Fazekas de St.Groth, 1950; Fazekas de St.Groth and Donnelley, 1950 a), and it is only those concerned directly with the titration of antibodies which are given here in any detail.

Collection of Blood.

Mice were bled from their tail veins, and approximately 0·2 ml. of blood collected in capillary tubes of 5 cm. length. Both ends of the tubes were sealed in a small flame. The serum was separated from the clot by centrifugation, after which the samples were stored at 4° C. To remove the serum, the tubes were cut at either end of the serum column, the half containing the clot was discarded, and the necessary volume of serum sucked directly into a calibrated Pasteur-pipette. Then the tubes, containing now only serum, were sealed again.

From animals killed in the course of the experiments, blood was collected from their severed art. subclavia into narrow precipitin tubes, incubated for 30 minutes at 37° C., spun, and the serum removed immediately from the deposit.

Since all titrations were done within 48 hours of obtaining the samples, no preservative was used, and the only measure taken to reduce bacterial growth was the storage of sera at 4° C.

Pre-treatment of Sera.

Shortly before the titrations, the sera were diluted 1:5 in normal saline and heated at 62° C. for 15 minutes in the water-bath. This treatment served the double purpose of (1) inactivating the complement, as well as (2) destroying the heat-labile nonspecific inhibitor interfering with anti-haemagglutinin titrations, and the nonspecific reacting substance giving false positive reactions in the complement fixation test (Donnelley, 1950).

The specific antibody titres are consistently reduced ($0·4 \pm 0·1$ $\log_2$ units) by this treatment. This reduction, however, is insignificant in tests where the positive titres vary between $\log_2$ 5·0 and 10·0, i.e. the reduction being of the order of less than 10 p.c., taken on a logarithmic scale, causes only a negligible loss in the sensitivity of the technique. On the other hand, the nonspecific inhibitor is reduced to such an extent that in the several thousand antibody titrations none of the nominally normal sera gave positive reactions in our starting dilution (1:5); similarly, sera of high specific antibody levels gave consistently negative results when tested against the heterologous antigen.

For a critical evaluation of the technique see the quoted paper by Donnelley (1950).

Collection of Bronchial Washings.

The method is described in the first paper of this series (Fazekas de St.Groth, 1950). In the case of lungs without areas of consolidation (after non-infective vaccines and in the early stages of experimental disease) only those fluids were used which did not contain red cells. Washings obtained from animals in the later stages of infection often contain traces of blood; this fact will be specified in the Tables below.

The bronchial washings were not inactivated, as preliminary experiments have shown that the samples were void of substances giving nonspecific reactions with either of the viruses used in the tests for antibody. Only the first washings were titrated, and the titres are not corrected for the "residual quantity" of fluid in the lungs. (The average correcting factor would be, with the volume of fluid used in the experiments, + 25 p.c., i.e. a titre of 16 would equal 20.) By abandoning this procedure the recorded readings give 25 p.c. lower values, and, as the serum titres were reduced by the same factor during heating, it is possible to compare directly the serum and bronchial antibody content.

Titration of Anti-haemagglutinin.

The tests were performed in round-bottomed tubes of 1·0 cm. internal diameter. Doubling dilutions of the samples were made in 0·25 ml. volumes of 0·5 p.c. fowl cells in normal saline. Only "lipid insensitive" cells (Stone, 1946) were used, as bronchial washings invariably contain a lipid agglutinin. One standard drop, approximately 0·03 ml., of saline containing 5 agglutinating doses of virus was added to each tube, the racks shaken, and the pattern of the settled cells read after 30 minutes' standing at room temperature. (An "agglutinating dose" in this test is defined as the amount of virus which causes partial agglutination of 0·25 ml. 0·5 p.c. fowl cells.) Partial agglutination was taken as the end-point, and the titres are expressed as the reciproçal of the serum dilution giving this pattern. Melbourne (A) and LEE (B) were the virus strains used in the tests, both in the active state.

The Complement Fixation Test.

The method of Donnelley (1950) was used; for full details see the original paper.

Sera were diluted by the drop technique, and equal volumes (2 standard drops—0·06 ml.) of serum, complement and antigen were mixed in this order in precipitin tubes (round bottom, 0·5 cm. internal diameter). The racks were incubated for 60 minutes at 37° C., then 2 standard drops of sensitized sheep cells added, and the degree of haemolysis read after further 30 minutes at 37° C.

The antigen was prepared from mouse lungs, and contained only the soluble "S30" component. 3 p.c. sheep cells sensitized with 4 units of haemolysin (rabbit serum) were used as indicator, and guinea-pig serum was diluted to contain three 50 p.c. haemolytic doses in the standard volume. 50 p.c. lysis was taken as the end-point, and the titres are referred to the initial dilution of serum, i.e. not to the final volume in the test.

EXPERIMENTS.

The Anti-haemagglutinin Response to Active Virus.

In the *first series of experiments* the animals were given different amounts of live virus by the nasal route.

Dilutions of recently harvested, high titre allantoic fluids were made up in sterile saline containing 10 p.c. horse serum; they ranged from 10^0 to 10^{-10}. 0·05 ml. volumes of these dilutions were instilled intranasally under light aether-chloroform (2:1) anaesthesia to large groups of six-weeks-old mice. Following instillation ten mice of each group were killed each day, their blood collected and bronchial washings taken, as described under Methods. All samples were titrated for anti-haemagglutinin; the values given in the following Tables are the geometric means of at least ten titrations. Since the mice were the same as those on which the experiments reported in the first two papers of this series were performed, the antibody response can be directly correlated with the changes there described.

The heavier inocula of live virus caused a typical disease in all mice, and since less than 10 p.c. survived the seventh day after administration of allantoic fluid dilutions up to 10^{-4}, these groups are not included in the following Tables. It should be noted that the sera of these animals did not contain measurable quantities of specific antibody if death occurred on the sixth day or earlier, and only very little (titres <20) on the seventh day.

The anti-haemagglutinin response to experimental infection with non-lethal doses of Melbourne (A) virus is shown in Table 1. For the sake of comparison, the degree (percentage) of lung consolidation on the seventh day is given for each experimental group.

TABLE 1.

Serum anti-haemagglutinin titres after experimental infection.
(Melbourne A virus.)

Dilution of inoculum	Average lung lesions on 7th day (p.c. consolidation)	Days after intranasal inoculation of virus												
		6	7	8	9	10	11	12	13	14	15	16	19	21
10^{-4}	67	—	19	39	78	170	141	256						
10^{-5}	43	—	4	14	117	109	207	228	290	127	167	151	160	116
10^{-6}	19	—	4	4	10	61	60	109	191	438	280	91	151	89
10^{-7}	11	—	3	5	8	19	10	22	19	78	23	44	23	8
10^{-8}	8	—	—	3		9	9	14	17	10	7	4	—	—
10^{-9}	0	—	—	—		—	—	—	—	—	—	—		—

Each figure represents the geometric mean of a group of ten mice. — = <3.

Over a wide range of infective inocula (10^{-4}–10^{-7}) the serum anti-haemagglutinin response is uniform, though the extent of lung lesions decreases progressively. This zone of maximum antibody response ended at the dilution 10^{-6} in our experiment; after this the average titres dropped rapidly, and in the group given a 10^{-9} dilution of allantoic fluid virus, there was no measurable antibody produced in the 21 days following intranasal instillation.

In all groups, irrespective of the infective inoculum, antibody appeared in the circulation between the seventh and eighth day, and after a period of roughly logarithmic increase reached its maximum round the twelfth day. Already by the end of the third week the serum titres were measurably lower.

The antibody titres within each group were widely scattered, and it was not uncommon to find sixfold differences—especially in the early days of the process—among the sera of mice which had received identical inocula at the beginning of the experiment. This variation is evident even from the averages recorded in the Table; no undue significance should be attached therefore to individual daily mean values. The data are amply sufficient to establish the general trend of the sequence, and comparison between groups will be made always on the grounds of at least five daily averages, by which means positive and negative deviations tend to cancel out.

The anti-haemagglutinin content of bronchial washings obtained from the same groups is summarized in Table 2.

Free antibody appears in the bronchi at the same time as in the circulation; its increase, climax and decrease run parallel to that of serum antibody.

If the blood volume of a seven-weeks-old mouse is taken as 1·9 ml., then the serum volume is roughly 1·0 ml., that is, four times the volume in which the antibody titrations were performed. The bronchi were washed with 0·5 ml. volumes of saline: twice the volume of the *in vitro* test. Thus, if the serum titres are multiplied by $4/2 = 2$, the two titres are directly comparable, and their ratio expresses the amount of antibody free in the bronchi as a fraction

of the serum antibody. This ratio, in form of percentage (100 × bronchial antibody per serum antibody), will serve in future as a characteristic of antibody distribution between the circulation and the site of possible infection.

TABLE 2.

Bronchial anti-haemagglutinin titres after experimental infection.
(Melbourne A virus.)

Dilution of inoculum	Average lung lesions on 7th day (p.c. consolidation)	Days after inoculation							
		6	7	8	10	12	14	16	21
10^{-4}	67	—	1·4	5·0	10·4	26·8			
10^{-5}	43	—	0·8	2·1	9·1	18·4	10·2	14·5	9·5
10^{-6}	19	—	0·7	1·4	9·1	14·6	28·0	9·5	8·0
10^{-7}	11	—	0·7	1·2	2·1	1·7	4·5	1·4	1·4
10^{-8}	8	—	0·6	0·7	0·6	1·2	1·1	0·9	0·6
10^{-9}	0	—	—	—	—	—	—	—	—

Each figure represents the geometric mean of a group of ten mice. — = $<0·5$.

The antibody "distribution coefficients" between days 10 and 21, as calculated from the data of Tables 1 and 2, are 4·4, 4·4, 5·4, 4·5, 6·8 for the groups 10^{-4} to 10^{-8}, respectively. They give an average of $5·1 \pm 0·41$, i.e. after intranasal infection with non-lethal doses of influenza virus 5 units of anti-haemagglutinin are free in the bronchi for every 100 present in the serum. This value appears to be characteristic of the convalescent stage after experimental influenza, as ten-thousandfold differences in the infective inoculum and more than tenfold variations in the mean antibody levels of the individual groups fail to cause any significant change in the distribution of antibody between the circulation and the site of infection. (Table 3).

TABLE 3.

Distribution of antibody after experimental infection.

$$\left(\text{Distribution coefficient} = \frac{100 \times \text{bronchial antibody titre}}{2 \times \text{serum antibody titre}}\right)$$

Dilution of inoculum	Days after inoculation							Mean ± standard error (between days 10 and 21)
	7	8	10	12	14	16	21	
10^{-4}	3·7	6·4	3·6	5·2				4·4 ± 0·6
10^{-5}	10·0	7·5	4·2	4·0	4·5	4·8	4·6	4·4 ± 0·06
10^{-6}	8·8	17·5	7·5	6·7	3·2	5·2	4·5	5·4 ± 0·69
10^{-7}	11·7	12·0	5·5	3·9	2·9	1·6	8·8	4·5 ± 1·11
10^{-8}	>10·0	11·7	3·3	4·3	5·5	11·3	>10·0	6·8 ± 1·42

Values calculated from data of Tables 1 and 2.

In the *second series of experiments* active virus was given by routes other than the nasal.

Virus-containing allantoic fluids were diluted in horse serum saline and 0·10 ml. volumes of the different dilutions given to groups of six-weeks-old mice either intraperitoneally or subcutaneously. Blood samples and bronchial washings were taken daily from five mice per group, and titrated for anti-haemagglutinin. Tables 4 and 5 show the average serum titres for the first three weeks of the experiment.

TABLE 4.

Serum anti-haemagglutinin titres after peritoneal vaccination with live virus.
(Melbourne A strain, titre 960.)

Dilution of inoculum	Days after inoculation													
	3	4	5	6	7	8	10	11	12	13	14	15	17	20
$10^{0.0}$	7	35	35	87	130	309	315	246	222	362	315	252	100	121
$10^{-0.5}$	—	7	33	65	48	96	299	207	127	260	362	172	107	106
$10^{-1.0}$	—	—	3	7	11	15	14	30	39	75	75	77	56	33
$10^{-1.5}$	—	—	3	4	5	15	18	19	5	21	38	19	17	10
$10^{-2.0}$	—	—	—	4	8	7	8	12	5	24	15	18	10	8

Each figure gives the geometric mean of a group of five mice. — = <3.

TABLE 5.

Serum anti-haemagglutinin titres after subcutaneous vaccination with live virus.
(Melbourne A strain, titre 960.)

Dilution of inoculum	Days after inoculation													
	3	4	5	6	7	8	10	11	12	13	14	15	17	20
$10^{0.0}$	—	—	4	17	18	15	34	38	29	43	152	123	31	69
$10^{-0.5}$	—	—	4	5	7	8	9	12	14	14	132	41	36	41
$10^{-1.0}$	—	—	—	4	8	10	9	16	8	12	44	44	28	10
$10^{-1.5}$	—	—	—	5	5	10	8	9	5	19	34	12	10	10
$10^{-2.0}$	—	—	—	—	3	3	5	5	8	13	19	29	9	9

Each figure gives the geometric mean of a group of five mice. — = <3.

It is seen that the antibody levels run parallel to the virus content of the inoculum, both in the case of peritoneal and subcutaneous vaccination. To produce identical serum titres 100,000 times more active virus is needed by the peritoneal and 1,000,000 times more by the subcutaneous route than when given intranasally.

There is also a remarkable difference in the time relations of the appearance of antibody. In the case of experimental infection antibody appeared on the seventh day. After peritoneal and subcutaneous vaccination with live virus (and as will be seen below, the same holds also for inactivated vaccines)

measurable amounts of anti-haemagglutinin are present on the third and fifth day respectively and there is a very rapid and steady increase in the following days, so that by the eighth day nearly maximal titres are reached. The significance of this consistent and striking difference will be discussed at the end of this paper. The changes in the antibody level during the second and third weeks are practically identical with those found after experimental infection: a short peak between the tenth and fourteenth days is followed by the gradual, slow decline.

TABLE 6.

Bronchial anti-haemagglutinin titres after peritoneal vaccination with live virus.
(Melbourne A strain, titre 960.)

Dilution of inoculum	Days after inoculation													
	3	4	5	6	7	8	10	11	12	13	14	15	17	20
$10^{0.0}$	—	—	—	—	0·5	0·6	0·7	0·7	0·9	1·6	1·3	1·5	1·6	1·1
$10^{-0.5}$	—	—	—	—	—	—	0·5	0·6	0·7	1·2	1·4	1·3	1·2	1·0
$10^{-1.0}$	—	—	—	—	—	—	—	0·5	0·5	0·8	1·1	0·9	0·7	0·5
$10^{-1.5}$	—	—	—	—	—	—	—	0·5	—	0·5	0·6	0·5	0·5	—
$10^{-2.0}$	—	—	—	—	—	—	—	—	—	0·5	0·5	—	—	—

Each figure gives the geometric mean of a group of five mice. — = <0·5.

TABLE 7.

Bronchial anti-haemagglutinin titres after subcutaneous vaccination with live virus.
(Melbourne A strain, titre 960.)

Dilution of inoculum	Days after inoculation													
	3	4	5	6	7	8	10	11	12	13	14	15	17	20
$10^{0.0}$	—	—	—	—	—	—	—	0·5	—	0·6	1·1	1·2	0·8	0·8
$10^{-0.5}$	—	—	—	—	—	—	—	—	—	0·5	1·0	1·1	0·9	0·8
$10^{-1.0}$	—	—	—	—	—	—	—	—	—	—	0·5	0·5	0·5	0·5
$10^{-1.5}$	—	—	—	—	—	—	—	—	—	—	0·5	0·5	—	0·5
$10^{-2.0}$	—	—	—	—	—	—	—	—	—	—	—	0·5	—	—

Each figure gives the geometric mean of a group of five mice. — = <0·5.

The bronchial anti-haemagglutinin titres are markedly lower than after intranasal infection. Antibody appears after a considerable lag, and in all instances in minute quantities only. The "distribution coefficients" are characteristic: on the average less than one two-hundredth of the serum antibody is free in the bronchi, the mean value over the period of the last ten days of the experiment being $0·4 \pm 0·07$ p.c. And this occurs in animals whose circulating antibody levels are as high as those found after experimental infection.

TABLE 8.

Distribution of antibody after peritoneal and subcutaneous vaccination with live virus.

$$\left(\text{Distribution coefficient} = \frac{100 \times \text{bronchial antibody titre}}{2 \times \text{serum antibody titre}}\right)$$

Site of inoculation	Dilution of inoculum	Days after inoculation										Mean ± standard error (between days 10 and 20)
		7	8	10	11	12	13	14	15	17	20	
Peritoneum	$10^{0.0}$	0·2	0·1	0·1	0·1	0·2	0·2	0·2	0·3	0·8	0·5	0·3 ± 0·08
	$10^{-0.5}$	—	—	0·1	0·1	0·3	0·2	0·2	0·4	0·6	0·5	0·3 ± 0·06
Subcutis	$10^{0.0}$	—	—	—	0·7	—	0·7	0·4	0·5	1·3	0·6	0·5 ± 0·14
	$10^{-0.5}$	—	—	—	—	—	1·8	0·4	1·3	1·2	1·0	0·6 ± 0·24

Values calculated from data of Tables 4, 5, 6 and 7.

Anti-haemagglutinin Response to Killed Virus.

Two types of inactivated virus were used in the following experiment. The "*formol vaccine*" was prepared from high titre allantoic fluid; 0·05 p.c. formaldehyde (i.e. 0·125 p.c. formol) was added and the mixture incubated at 37° C. for 12 hours. By this treatment infectivity was lost completely, the haemagglutinin titre and the capacity to bind specific antibody remaining unaltered; enzymic activity (as tested by the power to destroy the inhibitor in mucoids, or to elute from red cells) was only slightly reduced, by less than 25 p.c. "*Heated vaccine*" was prepared by heating the virus-containing allantoic fluids at 56° C. for 60 minutes. The resultant preparation proved to be non-infective when tested in eggs, lost approximately 50 p.c. of its haemagglutinin titre and antibody binding power; the vaccine had no measurable enzymic activity by the tests used: it did not elute spontaneously from red cells, nor did it destroy the inhibitory titre of mucoids in the Stone-test (Stone, 1949). In order to have identical amounts of antigen, the formol vaccine was diluted with normal saline to give the same haemagglutinin titre as the heated vaccine.

In the *first experiment* the vaccines were given by the nasal route. The technique was identical with that used in the case of active virus, and is described above. Both serum and bronchial anti-haemagglutinin titres were determined daily, in groups of ten mice. The results are summarized in Table 9.

After nasal vaccination with inactivated virus antibody appears in the circulation on the fifth and sixth day after heated and formol vaccines, respectively. The shape of the antibody curves is practically identical for the two types of vaccines, though heated virus appears to elicit a stronger serological response than formol vaccine when given nasally. This is reflected both by the earlier appearance of antibody in the circulation as well as in the respiratory tract, and by the higher maximum level between the tenth and fifteenth days. The average titres for this period are 35:70 in the serum, and 3·7:6·2 in the bronchi, that is, a roughly 1:2 ratio in favour of the heated vaccine.

The most striking feature of the results is the high antibody content of bronchial washings which is in sharp contrast to the relatively low serum titres. This is evident from the distribution coefficients which show 5 p.c. of

TABLE 9.

Anti-haemagglutinin response to nasal vaccination with inactive virus.

(Melbourne A strain, titre 500.)

Material tested	Type of vaccine	Days after inoculation														
		4	5	6	7	8	9	10	11	12	13	14	15	17	19	21
Serum	formol	—	—	3	15	12	20	24	35	42	30	45	36	34	30	30
	heated	—	5	12	27	21	35	60	72	81	64	80	64	51	30	42
Bronchial washings	formol	—	—	—	—	0·5	0·9	1·9	4·5	3·9	3·5	4·1	4·2	4·1	2·7	3·2
	heated	—	—	—	0·9	2·0	3·2	4·3	7·0	7·2	5·0	7·2	6·2	4·2	3·2	3·9

Each figure gives the geometric mean of a group of ten mice.
— = <3 in serum, and <0·5 in bronchial washings.

TABLE 10.

Anti-haemagglutinin response to peritoneal vaccination with inactive virus.

(Melbourne A strain, titre 500.)

Material tested	Type of vaccine	Days after inoculation													
		4	5	6	7	8	9	10	11	12	14	15	17	19	21
Serum	formol	27	28	35	71	92	141	203	176	246	198	212	172	164	120
	heated	—	24	32	50	64	86	115	155	217	232	172	203	145	155
Bronchial washings	formol	—	—	—	—	—	0·7	—	1·2	1·7	1·9	2·1	1·3	1·3	1·1
	heated	—	—	—	—	—	—	0·5	0·9	1·3	1·6	1·8	2·3	2·0	1·6

Each figure gives the geometric mean of a group of ten mice.
— = <3 in serum, and <0·5 in bronchial washings.

the serum antibody being present in the lower air passages. The values for the two vaccines do not differ significantly from each other, ("formol" $5 \cdot 2 \pm 0 \cdot 33$, "heated" $4 \cdot 5 \pm 0 \cdot 17$) nor from those found after experimental infection. The distribution coefficient of 5 appears to be constant for nasal (local) immunization, irrespective of whether the antigen was infective or inactivated by various methods.

In the *second experiment* 0·10 ml. of the vaccines was given intraperitoneally. The daily average anti-haemagglutinin titres are shown in Table 10.

The antibody response to inactivated vaccines after peritoneal administration is, to all appearances, identical with that found after active virus given by the same route (see Table 4 and 6). The slightly lower maximum levels can be accounted for by the lower initial agglutinin titres of these vaccines, as it will be recalled that heating destroys half of the antigen (haemagglutinin), and in order to have comparable amounts, the formol vaccine was diluted to the same final titre.

There is no appreciable difference between the response to the two vaccines; if any, formolized virus seems to be the better antigen after peritoneal administration. The average anti-haemagglutinin titres between the tenth and fifteenth days are 207:178 in the serum, and 1·38:1·22 in the bronchi for formol and heated vaccines, respectively. This apparently paradoxical "antigenic" behaviour of the two types of vaccines when given by different routes, will be discussed below.

Antibody in the bronchi (similarly to that demonstrated after active virus given peritoneally) appears after a lag of six days, and in very small quantities only, both in absolute values and as related to the serum titres. The average distribution coefficients ($0 \cdot 4 \pm 0 \cdot 06$, for both vaccines) show that $\frac{1}{250}$ of the circulating antibody only finds its way into the respiratory tract. This value is practically identical with that found in animals vaccinated with active virus by the peritoneal route.

The Complement-fixing Antibody.

All samples on which anti-haemagglutinin titrations were made were tested for the antibody against the soluble "S30" component of infected tissues, the "complement-fixing antibody" (CF-antibody). The complement fixation test of Donnelley (1950) is specific for this antibody, since the particulate viral antigen which would fix complement in the presence of its specific anti-haemagglutinin is carefully removed from the standard antigen used in the test, thus allowing the separate determination of the anti-S30, even in fluids which contain high concentrations of anti-haemagglutinin.

The results obtained in the group of experimentally infected mice (after *intranasal instillation of live virus*) are given in Table 11. The technical details of the experiment were described above, in connection with the determination of anti-haemagglutinin in the same group.

Complement-fixing antibody appears in the serum at the same time as the anti-haemagglutinin, the shape of the two antibody curves is also very much the same. Maximal titres develop in all mice which survive infection with dilutions up to 1 : 1,000,000 of the virus, and the antibody response ends at the same dilution (10^{-8}) as was found for the anti-haemagglutinin response.

TABLE 11.

Complement-fixing antibody titres in serum after experimental infection.
(Melbourne A virus.)

Dilution of inoculum	Days after intranasal inoculation											
	6	7	8	9	10	11	12	13	15	16	19	21
10^{-4}	—	0·5	1·0	5·0	5·1	5·2	4·9					
10^{-5}	—	—	0·4	4·6	5·0	5·5	5·5	3·9	4·7	4·6	4·6	5·3
10^{-6}	—	—	1·2	3·6	4·6	4·5	4·0	4·3	4·3	5·2	5·3	5·5
10^{-7}	—	—	1·0	0·3	3·5	3·7	2·4	3·2	3·0	2·4	3·2	—
10^{-8}	—	—	0·3		—	0·1	—	1·2	0·5	0·5	1·3	—
10^{-9}	—	—	—		—	—	—	—	—	—		—

Each figure gives the geometric mean of a group of ten mice.
Titres expressed in $\log_2$ units; — = <0.

The complement fixing antibody appears also in bronchial washings showing the same distribution between the circulation and the respiratory tract as the anti-viral antibody, so much so that a detailed description of the results seems superfluous.

In sharp contrast are the results obtained in the groups where active virus was given peritoneally or subcutaneously, or the inactivated vaccines by any of the three routes of administration. In neither case was there complement-fixing antibody detected, though the same groups contained high titre anti-haemagglutinin in their sera.

In rare cases, after peritoneal immunization with large doses of active virus, the animals also showed traces of complement-fixing antibody in their sera (individual titres <10, group averages <2). This finding, however, is not considered as characteristic for the respective group, since it is known from the work of the Henles (1946) that haemorrhages occur in the mucosae due to the toxic effect of large virus doses given peritoneally. This in turn gives the virus an opportunity of entering the bronchi, reaching the susceptible cells from their bronchial surface, and causing, incidentally, their infection. 10^9 times more virus is required to elicit comparable CF-antibody production when given intravenously or peritoneally, and any treatment capable of causing bronchial haemorrhages decreased this ratio proportionally to the extent of bleeding into the bronchi. Therefore, the exceptional appearance of complement-fixing antibody after extra-respiratory administration of live virus is attributed to an indirect, secondary type of bronchial inoculation of infective particles. The full experimental evidence for this interpretation will be published in a later communication.

DISCUSSION.

The main interest of the present study lies in the demonstration of marked variations in the serological response depending on the type of vaccines used and on the route of administration. Significant differences were shown both in regard to the quantity and distribution of antibody produced, as well as to the time relations of the process. Consequently, the serologic status of an indi-

vidual cannot be described by a single characteristic, and the use of the serum antibody level as sole criterion is clearly inadequate.

With influenza virus as antigen, the *distribution of antibody* between the circulation and the site of virtual infection was studied by comparing the antibody content of the serum and of bronchial washings, i.e. of the respiratory tract. The ratio of the two titres, expressed as percentage (100 × bronchial antibody/serum antibody) furnishes a convenient measure of this distribution. Such values were determined after administration by three different routes of live and inactivated virus. As established by the work of Fairbrother and Hoyle (1937), and repeatedly confirmed by others, it is only the anti-haemagglutinin which protects against infection, the antibody produced against the "S30" component of infected tissues (called CF-antibody in our papers) being devoid of such qualities. Therefore, in view of immunological implications, the distribution studies were made in greater detail on the anti-haemagglutinin.

Following experimental infection (intranasal instillation of live virus) antibody appears round the seventh day, simultaneously, both in the circulation and the respiratory tract. The ratio of the two titres is fairly constant throughout the period investigated, and gives 5·1 as distribution coefficient; that is, 5 units of antibody are free in the air passages for every 100 contained in the serum. Irrespective of the amount of virus inoculated, provided the infection is non-lethal, this value holds for the whole range over which antibody production was demonstrable, and thus can be taken as the distribution of antibody characteristic of the typical disease. Similarly high values of 5·2 and 4·5 were obtained after intranasal administration of killed (formolized and heated) virus vaccines, though the absolute amounts of antibody were considerably lower in these groups than in mice convalescent from experimental influenza.

Strikingly lower are the distribution coefficients after peritoneal or subcutaneous administration of either live or killed virus, irrespective of the type and quantity of antigen given. There is only 0·4 p.c. antibody present in the respiratory tract, twelve times less than after nasal (local) immunization.

The experimental findings are sufficient to establish that the distribution of antibody is governed by the route of vaccination, and is completely independent of both type and quantity of virus-antigen used. The mechanism by which such widely different distributions are brought about was specially studied, and will be published later in this series. Since different antibody concentrations at the site of possible infection have, obviously, a strong bearing on the prophylactic aspects of the serological response, a fuller appreciation of the phenomenon will be given, together with the relevant immunological data, in Paper IV of this series.

The *quantity of antibody* produced is a function of the quantity of antigen introduced parenterally into the animal organism. At first sight our results

seem to contradict this principle rather than confirm it. Yet, on further analysis, it becomes evident that the general law holds good for all of our findings. There are three discrepancies which call for some discussion.

(1) In experimental infection there is no correlation between the infective virus (antigen) dose and the amount of antibody produced, the serological response being uniform over a wide range of concentrations (10^{-4}–10^{-7}). However, such a correlation cannot be expected as the virus is multiplying after its introduction into the respiratory tract, and the final concentration in the lungs is practically identical over the range in question (Fazekas de St.Groth and Donnelley, 1950 a). Thus the antibody response is strictly proportional to the quantity of antigen finally present in the lungs of infected mice, not to the quantity introduced.

(2) The antibody response to identical amounts of antigen, when given by different routes, is different. This is seen e.g. in the experiment where inactive vaccines were given nasally or peritoneally. However, it has to be borne in mind that only that fraction of antigen is capable of eliciting antibody production which is given parenterally. In the case of peritoneal or subcutaneous vaccination all of the virus has an opportunity of coming into contact with antibody-producing cells. After intranasal instillation only 15–20 p.c. of the inoculum reaches the lungs (Fazekas de St.Groth, 1948 b; Fazekas de St.Groth and Graham, 1949), and has the chance of combining with and being taken up by cells. The rest is lost in the cavities lined with stratified epithelia. Such a difference in the immunizing dose fully accounts for the difference in the observed antibody response.

The importance of the uptake by cells of antigen is even more pronounced when the two types of killed vaccines are compared.

(3) The ratio of antibody produced to enzymically active ("formol") and inactive ("heated") vaccines varies with the site of administration. In the peritoneal cavity (and in the loose connective tissue of the subcutis) the formol vaccine seems to be slightly better antigen than the heated one. Given nasally, heated virus is definitely superior to the formolized. This paradox can be solved by considering the mechanism of nasal immunization. The inoculated virus will be adsorbed quantitatively to cells possessing receptors destructible by the viral enzyme (Fazekas de St.Groth, 1948 a), and will be taken up by *viropexis* (Fazekas de St.Groth, 1948 c; Fazekas de St.Groth and Graham, 1949). During this relatively slow process the enzyme-possessing formolized virus is capable of eluting from the cellular receptor, and might be mopped up by and eliminated with the secreted respiratory mucus (Burnet, 1948). Heated virus, incapable of elution, has a better chance of being taken up by the cell to which it is adsorbed, and thus will enter the organism in larger quantity. This hypothesis will be supported by further experimental evidence, and the rôle of *viropexis* in immunological phenomena demonstrated in later papers of this series.

The *time relations of the serological process* show an interesting correlation between the final level of antibody produced and the time of its appearance in the circulation. This is very clear in all experiments where active virus was given peritoneally or subcutaneously and in those with inactive vaccines, irrespective of the route of administration. In groups where antibody appears early, its final level surpasses that of groups where antibody appears later. The phenomenon was found regularly in each experiment, yet, in view of the marked variations in daily average antibody titres, it is not safe to attempt an exact quantitative formulation of this evident positive correlation.

The exception from the rule was the behaviour of the antibody curve after experimental infection. Though the final titres in these groups were the highest of all, antibody appeared the latest. Inocula of identical virus content when given peritoneally in the active state or nasally after having been inactivated, resulted in well measurable antibody production in 3–5 days. After experimental infection no antibody was found, either in the circulation or in the respiratory tract, before the seventh day. Still, it is difficult not to sustain the suspicion that antibody *was* produced even in these animals, but escaped detection for the first four days, as it was used up quantitatively to neutralize the antigen present in the lungs. There are distinct features indicating that this could be the case. For instance, the fact that infected lungs fix complement in the absence of antiserum from the third day onwards points to the presence of an antigen-antibody complex (Donnelley, 1950). Since the questions involved are closely connected with the development of lesions in infected lungs, their full discussion is deferred to the paper specially concerned with these problems.

The appearance of *complement-fixing antibody* (anti-S30) seems to be a characteristic of infection; it was never found after the administration by any route of inactive vaccines, but was demonstrable in every instance following nasal instillation of live virus. As a rare exception, complement-fixing antibody appeared after peritoneal injection of heavy doses (10^9 ID) of live virus. However, there are very good reasons to believe that even in this case the susceptible cells were infected via their bronchial surface. Full experimental evidence supporting this view will be published in Paper VIII. The presence of complement-fixing antibody is thus found as consistently, and appears to be as specific for influenza infection as the disappearance of virus receptors and inhibitors from the respiratory tract during the actual disease. The mere *presence* of influenza virus, live or inactive, in an organism gives rise only to anti-haemagglutinin production. The appearance of complement-fixing antibody points to the previous *multiplication* of virus in a susceptible tissue, that is, to past infection. It will be remembered that multiplication of virus and production of complement-fixing antigen were demonstrable also only after

nasal administration of live virus, i.e. after experimental infection (Fazekas de St.Groth and Donnelley, 1950 a).

All these findings indicate that the respiratory mucosa is the only site where a susceptible organism can be infected, and at the same time provide a convenient means of diagnosing influenza infection, manifest or subclinical, by the use of the complement fixation test, a method applicable also to work on immunized populations.

SUMMARY.

The antibody response of mice receiving graded doses of influenza virus (live or inactivated by formaldehyde or heat) was studied. Sera and bronchial washings were titrated daily for three weeks following the administration of virus by the nasal, peritoneal or subcutaneous route. The ratio of antibody contained in the circulation and at the site of virtual infection is expressed by the "*antibody distribution coefficient*": 100 × bronchial antibody/serum antibody.

After experimental infection (nasal instillation of live virus) anti-haemagglutinin is first detectable on the seventh day. Maximum antibody levels are reached round the twelfth day, and at the end of the third week there is already a noticeable drop in titres. The serological response is practically uniform in all survivors from infections with 0·1 to 100,000 ID_{50}. The amount of antibody free in the bronchi is on the average 5 p.c. of that contained in the serum. (Distribution coefficient = $5 \cdot 1 \pm 0 \cdot 41$).

Three days after peritoneal and five days after subcutaneous administration of live virus anti-haemagglutinin is detectable in the circulation, but only after a lag of 4–5 days in the respiratory tract. Maximum titres are reached between the tenth and twelfth days. The serological response is proportional to the immunizing dose; to produce identical serum titres approximately 10^5–10^6 times more virus is needed by these routes than when given nasally. Only $1/250$ of the antibody is free in the respiratory tract (distribution coefficient = $0 \cdot 4 \pm 0 \cdot 07$); the distribution is independent of the absolute amount of antibody present.

After nasal vaccination with inactivated virus, anti-haemagglutinin appears in the circulation on the 5–6th days, and reaches its maximum before the end of the second week. Roughly ten times more killed virus is needed to produce identical titres by this route than by the peritoneal. The antibody distribution coefficients are high: $5 \cdot 2 \pm 0 \cdot 33$ for formolized and $4 \cdot 5 \pm 0 \cdot 17$ for heated vaccines.

Inactivated virus given peritoneally elicits the same response as the same amount of live virus given by the same route. The distribution coefficient is $0 \cdot 4 \pm 0 \cdot 06$ for both types of killed vaccine.

Complement-fixing antibody ("anti-S30") is detectable only after experimental infection of mice, the time curves and the distribution coefficients showing the same trend as the anti-haemagglutinin of the respective groups. No complement-fixing antibody is produced after peritoneal or subcutaneous administration of live virus, or after vaccination with killed virus by any of the three routes.

Quantitative and qualitative aspects of the immunization process are discussed in the light of these findings, and the importance of the route of entry of the immunizing antigen is emphasized. The conclusion is reached that the serum antibody level in itself is insufficient to describe the serological state of an individual, as it gives no information in regard to the concentration of antibody at the site of possible infection.

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STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

IV. THE PROTECTIVE VALUE OF ACTIVE IMMUNIZATION

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As shown in the previous paper (Fazekas de St.Groth and Donnelley, 1950 b), identical amounts of vaccine when given by different routes caused marked differences both in the amount and the distribution of antibody produced. Judged by the usual criterion, the serum antibody level, peritoneal vaccination should be the most and intranasal the least efficient method. However, the opposite conclusion might be reached on purely theoretical grounds, as one would suppose that only that fraction of antibody is capable of preventing infection, which meets and neutralizes the virus before it could enter a host cell. Hence the measure of immunity would depend more on the amount of antibody free in the respiratory tract than on that contained in the circulating blood.

With the data of our previous paper in hand, in an experimental approach, we tested the resistance to infection of mice vaccinated by different methods and whose serological status was fully known, in regard both to quantity and distribution of antibody. To substantiate the validity of conclusions reached in the first paper of this series (Fazekas de St.Groth, 1950), most groups were also challenged with the heterologous type of virus, by which means the non-specific resistance could be measured.

MATERIALS AND METHODS.

Only techniques with special bearing on the assay of protection against influenza infection will be described here; details about other materials and methods will be found in the first three papers of the series.

Vaccines.

The Melbourne strain of influenza A virus, and the LEE strain of influenza B were used in the experiments, both in the form of infected allantoic fluid. Only fluids of high haemagglutinin titre and free of bacterial contamination were used. They are referred to as *active* virus, i.e. infective when tested in eggs or mice.

Two types of inactive virus (non-infective) were used. The *formol vaccine* was prepared by adding 0·05 p.c. formaldehyde (0·125 p.c. formol) to active virus-containing allantoic

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fluids, and incubating the mixture at 37° C. for 12 hours. The haemagglutinin titre remained unchanged after this treatment, enzymic activity was reduced by less than 25 p.c., as found in the Stone-test (Stone, 1949). *Heated vaccine* designates virus-containing allantoic fluids which were heated at 56° C. for 60 minutes. More than 99 p.c. of the enzymic activity of these preparations was lost, and the haemagglutinin titre dropped by approximately one $\log_2$ unit.

Vaccination.

For *intranasal instillation* the mice were lightly anaesthetized with aether-chloroform (2:1). Special care was taken to perform the instillation at the right degree of anaesthesia since too lightly anaesthetized mice do not breathe through their noses and thus do not take the inocula, too deeply anaesthetized ones pant and blow bubbles which might cause a considerable loss in the quantity of fluid that reaches the lungs. This would not be a source of significant error in the case of infectivity titrations where the challenging inocula were diluted in tenfold steps. In vaccination with inactive virus, however, the quantity of antigen administered is of prime importance, and even a 50 p.c. loss could be misleading. Yet, with necessary caution these pitfalls can be avoided, and as found in previous studies (Fazekas de St.Groth, 1948; Fazekas de St.Groth and Graham, 1949), the volume of fluid which reaches the lungs varies within narrow limits only, and is consistently 15–20 p.c. of the volume given in the original inoculum.

For the *intraperitoneal inoculation* of vaccines, the animals were not anaesthetized. 0·10 ml. volumes were given from a tuberculin syringe into the right inguinal region. The leaking out of the inoculum was avoided by leading the needle for approximately 15 mm. through the connective tissue and muscles of the abdominal wall before piercing the peritoneum. *Subcutaneous inoculations* were given into the loose connective tissue of the right lumbar region. The skin was lifted and, to avoid leakage, the needle introduced for about 20 mm. before the inoculum (0·10 ml.) was injected.

Infectivity Titration.

Dilutions of active virus were made up in horse serum saline (10 p.c. horse serum in normal saline, sterile). 0·05 ml. volumes of the dilutions were given intranasally to groups of five mice. The technique of inoculation was the same as that described above in connection with nasal vaccination. Following inoculation, the individual groups were kept in separate tins, and observed for a week. All mice dying during this period were autopsied: the dorsal part of the chest wall was removed and the consolidation of the lungs noted. In no instance was specific death observed before the third day, and all mice dying of experimental influenza showed at least 90 p.c. of their lungs consolidated; in 96/100 cases complete consolidation was found. Seven days after intranasal instillation of the challenging dose, all mice were killed by over-anaesthetizing with the aether-chloroform mixture. There was ample opportunity to establish that the anaesthetic did not cause non-specific lesions, and the haemorrhages occurring exceptionally in lungs of mice killed by this method were easily distinguishable from the plum-coloured, consolidated areas of specific influenza lesions. Degrees of consolidation are designated as follows: 100 p.c. = 4, 75 p.c. = 3, 50 p.c. = 2, 25 p.c. = 1; specific death on the sixth day is given the value of 4·5, on the fifth day or earlier 5·0. The arithmetic mean of individual lesions is taken as the "average lung lesion" of a group.

Assessment of "Protection Values", and Discussion of the Method.

The infectivity of any given virus can be measured by the highest dilution in which it is capable of causing symptoms, or more accurately, by the dilution which causes a standard type of response in half of the animals tested. A convenient measure for influenza virus is the dose which causes 50 p.c. lung consolidation in mice, and infective materials are generally

compared on this basis. Consequently, the simplest method of determining protection would be to measure the dose of virus which causes 50 p.c. lung consolidation in vaccinated mice, and to compare this value with that found for normal mice. The ratio of the two end-point dilutions should express the degree of protection, that is, the degree to which the apparent infectivity of the virus is reduced in immunized animals.

TABLE 1.

Evaluation of the infectivity test I.

Immunization: ***nasally*** 0·05 ml. active LEE (B) virus diluted 10^{-5}; ***peritoneally*** 0·10 ml. active LEE (B) virus, undiluted.

Challenge: 11 days after immunization, 0·05 ml. of active LEE (B) virus intranasally; dilutions 10^{0} to 10^{-7}.

Experimental readings.

Dilution of challenging inoculum	Nasal immunization		Peritoneal immunization		Controls (not immunized)	
	Lesions	Average	Lesions	Average	Lesions	Average
10^{0}	1·0, 0·3, 0·2, 0·0, 0·0	0·30	†6, 2·0, 2·5 3·2, 3·0	3·04	†5, †5, †6, †6, †7	4·60
10^{-1}	0·2, 0·2, 0·0, 0·0, 0·0	0·08	†6, 1·5, 1·5 1·5, 2·0	2·20	†5, †6, †7, †7, †7	4·30
10^{-2}	0·2, 0·0, 0·0, 0·0, 0·0	0·04	2·5, 2·0, 0·8 2·5, 0·2	1·60	†5, †6, †7, †7, 2·5	4·00
10^{-3}	0·2, 0·0, 0·0, 0·0, 0·0	0·04	3·5, 1·5, 1·5, 0·5, 0·0	1·40	†5, †6, †7, 2·0, 1·0	3·30
10^{-4}	0·0, 0·0, 0·0, 0·0, 0·0	0·00	0·5, 0·5, 0·8, 0·2, 0·2	0·44	3·0, 2·5, 2·0 1·5, 0·2	1·84
10^{-5}	0·0, 0·0, 0·0, 0·0, 0·0	0·00	1·0, 0·3, 1·0, 0·2, 0·0	0·50	1·5, 1·0, 1·5 2·0, 1·0	1·40
10^{-6}					0·5, 2·5, 0·8 0·5, 0·3	0·92
10^{-7}					1·0, 0·8, 0·2 0·5, 0·8	0·66

The mice used in the tests were 6-weeks old at the time of immunization.

At the beginning of our study we intended to use this method; however, already after the first experiment it proved to be inadequate for this purpose. As will be seen from the Tables below, in many of the vaccinated groups even the most concentrated inocula failed to cause 50 p.c. lung consolidation, and the end-point could have been determined only by extrapolation, an exceptionally unreliable method when the experimental points fall on the flat ends of the sigmoid dose-response curve. Also, even in those groups where they were not missed, the end-points were never sharp. This is well accounted for by the two-dimensional variation in our tests: there is a horizontal factor—the response of mice to the vaccines, and a vertical one—their response to the different infective inocula. The importance of this aspect is clearly

seen from the fact that the non-immunized control animals give sharp infectivity end-points (only vertical variation!), a parallel of which was never found in groups of vaccinated mice, provided there was any protection afforded by vaccination.[2]

The requirements of a reliable method would be (1) to avoid the need of extrapolation, (2) to transform the sigmoid dose-response curve to a straight line, in order to express differences as linear functions, and (3) in view of the marked variations, to use a larger number of points for the determination of each curve.

These demands are met by the following method, which will be demonstrated on the first set of experimental results of this paper taken from Table 4. In the actual experiment, the protective value of mild experimental infection and of parenteral vaccination with live virus was determined. Mice were given 10^{-5} dilution of active LEE virus nasally or undiluted LEE virus peritoneally. Eleven days later these two groups and one containing non-immunized normal mice were challenged with dilutions of active LEE virus. The full experimental results are given in Table 1.

It can be seen that the controls give the expected answer: the dose-response curve fits well with the integrated normal curve, and the variations within the individual groups are of the magnitude usual for such tests. The group of peritoneally immunized mice gives irregular results: there are considerable variations within practically all sub-groups. To impress the uninitiated, the 50 p.c. end-point could be determined by force, but its statistical validity would be very restricted indeed. None of the thirty mice of the first group (after nasal immunization) showed 50 p.c. lung consolidation, and there is no reliable method of even as much as attempting to give any estimate of the 50 p.c. end-point.

We, therefore, took the integrated response of the whole group of animals to a standard series of infective inocula as the basis of our calculations. In order to obtain additive values for the average group-lesions, the sigmoid dose-response curve had to be transformed to a straight line. This was achieved by the probit-method of Bliss (1935).

The maximum response (specific death on the fifth day or earlier) carries the value of 5·0 on our arbitrary scale of evaluating lung lesions. This will be taken as 100 p.c., hence by multiplying the group averages by 20, the results are expressed as percentage of the maximum. The next step is to look up the probit values corresponding to each per cent. value. This will be found in any recent book on statistics; we used the Statistical Tables of Fisher and Yates (1943). The process of transforming the actual readings to probits can be followed in Table 2.

The sum of the six average-probit-lesions (10^{0}–10^{-5}) gives the "total lung lesion", ΣL, of a group of 30 mice. In the protected groups the total lesions are lower than in the normal controls, this is shown in Table 3. The difference in ΣL values which would be equivalent to a tenfold reduction in infectivity, can be determined from the controls, where the infectivity titrations were carried two steps further (to 10^{-7}), thus providing three sets of "total lung lesions" with known tenfold reductions from one group to the other. In

[2] This difference in variation was clearly recognised in a recent publication by W. and G. Henle (J. Immunol., 59, p. 45, 1948).

TABLE 2.

Evaluation of the infectivity test II.

Transformation of average lung lesions to probits.

Groups	Dilution of challenging inoculum	Average lung lesions expressed in		
		Arbitrary units (Table 1)	Percentage	Probits
Nasally immunized	10^0	0·30	6·0	3·45
	10^{-1}	0·08	1·6	2·86
	10^{-2}	0·04	0·8	2·59
	10^{-3}	0·04	0·8	2·59
	10^{-4}	0·00	0·0	0·00
	10^{-5}	0·00	0·0	0·00
Peritoneally immunized	10^0	3·04	60·8	5·27
	10^{-1}	2·20	44·0	4·85
	10^{-2}	1·60	32·0	4·53
	10^{-3}	1·40	28·0	4·42
	10^{-4}	0·44	8·8	3·65
	10^{-5}	0·50	10·0	3·72
Control (not immunized)	10^0	4·60	92·0	6·41
	10^{-1}	4·30	86·0	6·08
	10^{-2}	4·00	80·0	5·84
	10^{-3}	3·30	66·0	5·41
	10^{-4}	1·84	36·8	4·66
	10^{-5}	1·40	28·0	4·42
	10^{-6}	0·92	18·4	4·10
	10^{-7}	0·66	13·2	3·88

TABLE 3.

Evaluation of the infectivity test III.

Computation of protection values (P).

		Nasal	Peritoneal	Controls		
		10^0–10^{-5}	10^0–10^{-5}	10^0–10^{-5}	10^{-1}–10^{-6}	10^{-2}–10^{-7}
Probit-lesions (from Table 2)	L	3·45	5·27	6·41		
		2·86	4·85	6·08	6·08	
		2·59	4·53	5·84	5·84	5·84
		2·59	4·42	5·41	5·41	5·41
		0·00	3·65	4·66	4·66	4·66
		0·00	3·72	4·42	4·42	4·42
					4·10	4·10
						3·88
Total lung lesions	ΣL	11·49	26·44	32·82	30·51	28·31
Difference from control	$d = \Sigma L_{\text{cont.}} - \Sigma L_x$	21·33	6·38	0·00	2·31	2·31 + 2·20 $d_{10} = 2·255$
Reduction in lesions	$\log P = \frac{d_x}{d_{10}}$	9·45	2·84	0·00	1·04	2·00
Protection	$P = \text{antilog}_{10}(\log P)$	3×10^9	690	1	11	100

this experiment this reduction (d_{10}) is 2·255. The difference between the total lung lesions of the control group and of any given experimental group, divided by the d_{10} value, gives the degree of protection on a $\log_{10}$ scale.

$$\Sigma L_{\text{control}} - \Sigma L_x = d_x$$

$$\frac{d_x}{d_{10}} = \log P$$

The antilog of this gives the "protection value", P, the factor by which the infectivity of a standard virus preparation is reduced in a group of animals when compared with a set of normal controls. In our example, for instance, $P = 690$ for the peritoneally vaccinated groups means that the challenging virus proved to be 690 times less infective in this group, or conversely, that these animals are 690 times more resistant to infection with LEE virus than are non-immunized mice.

It was necessary to delimit the range of responses for which the probit-standard error could be considered as a constant. The statistical treatment of data from a large series of protection tests showed that the precision of the assays was such that the error of the P-values was found to be quite uniform between log P 0·0 and 4·0, giving $\pm$ 0·23 standard deviation on a $\log_{10}$ scale. Therefore between 1 and 10,000-fold protection equal weights are assigned to all points, and the difference of 1·0 between two log P values (tenfold difference) is significant with a probability of 97/100. Because of the appearance of groups with zero response (no lesions), in the range of higher protection (log $P > 4$) the error of the estimate is proportional to the value of P; thus in groups of higher P-values the minimum requirements of significance have to be elevated accordingly.

All Tables of this and following papers will give the directly determined total lung lesions (ΣL) of each experimental group, together with the difference of this value (d_x) from that found in the group of normal control mice. These data and the value of d_{10}, the equivalent of the average tenfold reduction in infectivity, are sufficient to compute the protection values (P), and to establish all our experimental findings. P, the protection value, characterizes the immunological and not the serological state of a group of animals, and is—as will be shown below—a function of the amount and type of antigen given and of the mode of administration. None of these independent variables can replace P in itself, though from the knowledge of all the three the protection value can be predicted.

EXPERIMENTS.

Protective Value of Immunization with Active Virus.

The *first group* of mice was inoculated intranasally with live virus. Since this method of inoculation results in experimental infection, the allantoic fluids were diluted 10^{-5} in horse serum saline to obtain a concentration of virus which would infect all mice but kill less than 10 p.c. of them. This aim was achieved with the LEE (B) strain which caused an average of 24 p.c. lung consolidation on the seventh day with none of the mice dying during the first

two weeks of the experiment. A similar dilution of the Melbourne (A) strain, however, proved to be too infective, the original allantoic fluid having an exceptionally high infectivity titre for mice ($ID_{50} = 10^{-6.2}$). As more than 40 p.c. of the animals died within ten days after instillation, and the average lung consolidation on the seventh day was 64 p.c., this half of the experiment had to be repeated. Consequently the results given in Table 5 are not those of a single experiment but of two, in which both the immunizing and challenging virus differed. Yet, this is no cause of error since the challenging inocula gave practically identical "total lung lesions" in the control groups, and the immunizing dilution given nasally (10^{-6}) killed 2 p.c. of the mice in fourteen days with 31 p.c. average lung consolidation on the seventh day, a result closely similar to that obtained in the LEE-immunized group.

The challenging dose of virus was given 11 days after immunization, in order to infect the animals during the period when antibody levels are maximal, both in the serum and in the respiratory tract. As shown in our previous paper (Fazekas de St.Groth and Donnelley, 1950 b), this range extended over the tenth to sixteenth days after immunization, thus covering the first five days of the infectivity titration.

The evaluation of lung lesions in experimentally infected groups did not cause any difficulty, because 18 days after the first infection the consolidated areas caused by the immunizing dose are of greyish colour and transparent due to resorption of the extravasate, fibrosis and progressing reorganisation; they cannot be confused with the sharply circumscribed, plum-coloured areas of recent lesions due to the challenging inoculum given seven days before.

The *second group* of mice was given active virus by the peritoneal route. 0·10 ml. volumes of undiluted allantoic fluid were injected, and the animals challenged with dilutions of active virus on the eleventh day after vaccination. No deaths occurred in this group, nor were any lung lesions observed on the seventh or eleventh day after immunization.

The *third group* of mice received live virus subcutaneously. The inoculum was the same as in the peritoneally immunized group: 0·10 ml. of undiluted allantoic fluid. Again no deaths and no lung lesions were seen in the group.

At the time of challenging, on the eleventh day, ten mice of each group were killed, their blood collected and bronchial washings taken. All samples were titrated individually against both of the challenging viruses (LEE and Melbourne). Tables 4 and 5 give the result of this experiment.

TABLE 4.

Protective efficiency of vaccination with active virus.
Challenging strain: LEE (B).

Vaccination		Antibody against LEE		Infectivity test			Protection
virus	route	serum	bronchi	ΣL	d_x	log P	(P)
	nasal*	164	19	11·49	21·33	9·45	3×10^9
LEE (B)	perit.	128	0·9	26·44	6·38	2·84	690
titre: 640	subcut.	34	—	28·90	3·92	1·74	47
MEL (A)	perit.	—	—	32·55	0·27	0·12	1
titre: 1,200	subcut.	—	—	33·52	—0·70	—0·31	—2
nil					$d_{10} =$		
(control)	—	—	—	32·82	2·255	0·00	1

— = <5 in serum and <0·5 in bronchial washings. * = dilution of 10^{-5}.

TABLE 5.

Protective efficiency of vaccinations with active virus.

Challenging strain: Melbourne (A).

Vaccination virus	route	Antibody against MEL serum	bronchi	Infectivity test ΣL	d_x	log P	Protection (P)
MEL (A) titre: 1,200	perit.	280	1·4	30·31	6·86	3·19	1,550
	subcut.	58	0·5	32·30	4·87	2·27	186
	nasal*	—	—	36·38	0·79	0·37	2
LEE (B) titre: 640	perit.	—	—	37·62	—0·45	—0·21	—2
	subcut.	—	—	35·60	1·57	0·73	5
nil (control)	—	—	—	37·17	d_{10} = 2·15	0·00	1
MEL (A) titre: 960	nasal§	231	23	11·82	24·25	11·95	9×10^{11}
nil (control)	—	—	—	36·07	d_{10} = 2·03	0·00	1

— = <5 in serum and <0·5 in bronchial washings. * = diluted 10–5. § = diluted 10–6.

The first striking feature of the Tables is the complete lack of cross-protection against the heterologous type of virus in animals highly immune against the strain they were vaccinated with. The second is the immense differences between the efficiency of active virus as vaccine when given by the nasal route and when inoculated peritoneally or subcutaneously. Mild experimental infection protects a million times better than extra-respiratory vaccination; of the two types of parenteral administration the peritoneal route is decisively superior to the subcutaneous.

The lack of correlation between the serum anti-haemagglutinin titres and the protection values is evident; on the other hand, this single experiment would be sufficient to reveal the intimate relationship between the amount of antibody free in the bronchi and the resistance to infection of the respective groups of animals. All experiments below add further evidence to this point, the significance of which will be discussed at the end of the paper.

The Protective Efficiency of Formolized Vaccines.

In the following experiments the vaccines were used in the undiluted form, their haemagglutinin titres are shown in the Tables. 0·05 ml. volumes were instilled intranasally, and 0·10 ml. injected peritoneally or subcutaneously. None of the animals died during the first fortnight after vaccination, and no lung lesions were seen on the seventh and eleventh days. All groups were challenged with dilutions of active virus eleven days after immunization, that

is, at the time when antibody attained its highest concentration both in the circulation and in the respiratory tract (see paper III). Ten mice of each group were killed at this time, and their sera and bronchial washings titrated for anti-haemagglutinin. Tables 6 and 7 give the results.

TABLE 6.

Protective efficiency of vaccination with formolized virus.
Challenging strain: Melbourne (A).

Vaccination virus	route	Antibody against MEL serum	bronchi	Infectivity test ΣL	d_x	log P	Protection (P)
MEL (A) titre: 1,000	nasal	39	2·3	28·96	7·11	3·50	3,160
	perit.	141	1·1	30·83	5·24	2·58	380
	subcut.	27	0·4	32·11	3·96	1·95	89
LEE (B) titre: 640	nasal	—	—	35·28	0·79	0·39	2
	perit.	—	—	35·93	0·14	0·07	1
nil (control)	—	—	—	36·07	d_{10} = 2·03	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

TABLE 7.

Protective efficiency of vaccination with formolized virus.
Challenging strain: LEE (B).

Vaccination virus	route	Antibody against LEE serum	bronchi	Infectivity test ΣL	d_x	log P	Protection (P)
LEE (B) titre: 640	nasal	31	2·7	24·13	7·50	3·25	1,780
	perit.	140	0·7	27·27	4·36	1·89	78
MEL (A) titre: 1,000	nasal	—	—	30·62	1·01	0·44	3
	perit.	—	—	32·25	—0·62	—0·27	—2
	subcut.	—	—	32·37	—0·74	—0·32	—2
nil (control)	—	—	—	31·63	d_{10} = 2·305	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

As in the previous experiment, the lack of cross-immunity between the two types of virus is evident once more. The protection afforded by parenteral vaccination is much the same as that after the administration of active virus by the same route, peritoneal immunization being again superior to subcutaneous. Nasal vaccination with formolized virus gives better protection than the double amount given parenterally. This is especially clear in the experiment with

the LEE strain, the difference being highly significant; with the Melbourne strain the difference is less marked but still significant. This discrepancy with the two types of virus was found consistently in each experiment where inactive vaccines with full enzymic activity were given nasally. The phenomenon was further investigated, and as it has a very direct bearing on the rôle of the susceptible cell in the immunization process, it will be dealt with in a later paper of this series, specially concerned with this problem.

The comparison of the nasally and parenterally immunized groups shows again the absence of correlation between serum antibody levels and protection; the positive correlation between resistance to homologous infection and the antibody content of bronchial washings is equally clear and impressive.

The Protective Efficiency of Heated Vaccines.

During the destruction of infectivity by heating (60 minutes at 56° C.) approximately 50 p.c. of the haemagglutinin is also destroyed. As shown in paper III, the serological response to these vaccines is accordingly lower, the drop in antigenic property running parallel to the drop in haemagglutinin content. However, the vaccine is not equivalent to a mere dilution of e.g. a formol-inactivated vaccine: it has no enzyme activity *in vitro*, and behaves quite differently from the formol vaccine *in vivo*.

The technicalities of vaccination were the same as described above in connection with the formol vaccine. 0·05 ml. volumes were instilled nasally and 0·10 ml. volumes injected peritoneally or subcutaneously. No deaths or pathological signs due to immunization were observed. Challenging inocula of live virus were given on the eleventh day and at the same time the average serum and bronchial antibody level of each group determined. These data and the results of the infectivity titration are given in Tables 8 and 9.

TABLE 8.

Protective efficiency of vaccination with heated virus.
Challenging strain: Melbourne (A).

Vaccination		Antibody against MEL		Infectivity test			Protection
virus	route	serum	bronchi	ΣL	d_x	log P	(P)
	nasal	64	4·3	28·84	9·45	3·96	9,120
MEL (A)	perit.	112	0·8	29·38	4·91	2·04	110
titre: 520	subcut.	39	—	30·75	3·54	1·47	29
	nasal	--	—	32·75	1·54	0·64	4
LEE (B)	perit.			33·86	0·43	0·18	2
titre: 300	subcut.	—	—	34·51	—0·22	—0·09	1
nil (control)	—	—	—	34·29	d_{10} = 2·41	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

TABLE 9.

Protective efficiency of vaccination with heated virus.
Challenging strain: LEE (B).

Vaccination virus	route	Antibody against LEE serum	bronchi	Infectivity test ΣL	d_x	log P	Protection (P)
	nasal	24	2·3	25·69	6·60	2·81	645
LEE (B)	perit.	98	—	28·97	3·32	1·42	26
titre: 300	subcut.	30	—	30·44	1·85	0·79	6
	nasal	—	—	32·55	—0·26	—0·11	1
MEL (A)	perit.	—	—	31·33	0·96	0·41	3
titre: 520	subcut.	—	—	33·13	—0·84	—0·37	—2
nil (control)	—	—	—	32·29	d_{10} = 2·34	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

The results closely parallel those obtained with formolized vaccines: there is no cross-protection, peritoneal immunization is more efficient than subcutaneous, and nasal vaccination is superior to both. The protection values are positively correlated with the bronchial antibody titres, and vary independently from the serum titres.

There is, however, one striking difference: the Melbourne strain, given nasally, affords better protection than the LEE strain, as judged by the difference between peritoneal and nasal P-values for the two viruses. This is the reverse of the finding with enzyme possessing vaccines, and will be analysed in a later communication (Paper X).

The Correlation Between the Immunizing Dose and the Protection Afforded.

In the previous paper it has been shown that the amount of antibody produced is a direct function of the quantity of antigen administered. By varying the immunizing dose the antibody content of the serum and bronchial washings varied within wide limits, yet the ratio of the two titres remained unchanged, that is, the distribution coefficient was found to be constant. On these grounds, since protection runs parallel to the antibody content of the respiratory tract, one would expect a positive correlation between the quantity of antigen given by a certain route and the resulting resistance to homologous challenge. Furthermore, within one type of immunization protection should be correlated with both the bronchial *and* serum antibody levels, as the ratio of the two latter is fixed. This assumption was subjected to experimental test with each route of vaccination used in our studies.

Groups of mice were given inocula of formolized Melbourne virus, 0·05 ml. volumes intranasally and 0·10 ml. volumes peritoneally or subcutaneously. Five different dilutions of the vaccine were made in normal saline in 0·5 $\log_{10}$ steps (1:1, 1:3·16, 1:10, 1:31·6, 1:100). The technique of administration was the same as in experiments above. Eleven days after immunization the mice, together with a group of untreated controls, were challenged with dilutions of active Melbourne virus. The results of the protection-tests are given in Table 10.

TABLE 10.

Correlation between immunizing dose and protection.
Vaccine: formolized Melbourne (A) virus, titre: 960.
Challenging strain: Melbourne (A) virus.

Dilution of vaccine	Antibody against MEL		Infectivity test			Protection
	serum	bronchi	ΣL	d_x	log$P$	(P)
Nasal vaccination.						
10^0	98	6·4	26·44	9·99	4·15	14,100
$10^{-0.5}$	61	4·9	27·83	8·60	3·57	3,720
$10^{-1.0}$	48	3·8	29·02	7·41	3·08	1,200
$10^{-1.5}$	19	1·1	31·64	4·79	1·99	98
$10^{-2.0}$	8	—	33·29	3·14	1·30	20
Peritoneal vaccination.						
10^0	167	1·1	31·14	5·29	2·19	155
$10^{-0.5}$	124	0·7	31·98	4·44	1·84	69
$10^{-1.0}$	42	—	32·76	3·67	1·52	33
$10^{-1.5}$	17	—	34·23	1·80	0·75	6
$10^{-2.0}$	12	—	35·80	0·63	0·26	2
Subcutaneous vaccination.						
10^0	67	0·6	32·21	4·22	1·75	56
$10^{-0.5}$	41	—	32·98	3·45	1·43	27
$10^{-1.0}$	34	—	34·62	1·81	0·75	6
$10^{-1.5}$	16	—	36·05	0·38	0·16	1
$10^{-2.0}$	—	—	36·91	—0·48	—0·20	—2
Control.						
No vaccine	—	—	36·43	d_{10} = 2·41	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

The prediction was fully verified by the experiment. The positive correlation between the quantity of immunizing antigen and the degree of protection is evident in all groups; the developed resistance is proportional, within each separate group, to the amount of both bronchial and serum antibody. The economy of the higher distribution coefficients shows up clearly when the results of nasal vaccination are compared with those of the extra-respiratory routes of administration; significantly lower total antibody content gives comparable protection values, as the immunologically available (bronchial) antibody levels are similar in spite of marked differences between serum antibody titres.

DISCUSSION.

A direct continuation of the previous paper, the present study was concerned with the resistance of vaccinated mice to influenza virus infection. In order to obtain reproducible quantitative data, an improved method of assaying resistance has been devised, and the results correlated with those obtained by serological methods (Fazekas de St.Groth and Donnelley, 1950 b).

From the investigation of virus receptors and inhibitors in the respiratory tract it was concluded that following influenza infection or vaccination with a single type of virus there is no change in the behaviour of susceptible cells which would render them refractory to infection, nor is there any non-specific inhibitor produced besides the antibody homologous to the immunizing virus (Fazekas de St.Groth, 1950). Hence, there should be no non specific resistance to heterologous challenge after either infection or vaccination with one type of influenza virus. It was realized, however, that this evidence remains circumstantial and incomplete as long as straight infectivity tests have not been applied. Therefore the vaccinated animals were also challenged with the heterologous type of virus, and their infectibility compared with that of untreated, normal controls. The experiments gave a clear-cut answer to the question: in the absence of specific antibody none of the mice showed signs of increased resistance, irrespective of whether they had been infected or vaccinated previously with an unrelated strain of virus.

With the homologous virus as challenging inoculum there is an excellent correlation between circulating antibody level and resistance to infection, provided one separates sharply the animals immunized by the nasal route from those immunized parenterally. Since intranasal instillation is not ordinarily used, this would support the generally expressed view that serum antibody level gives a rough measure of immunity expected after vaccination against influenza.

However, the present experiments show clearly that this correlation is essentially indirect. A useful reference in the study of different vaccines given by the same route, the serum titres tell but little about the specific resistance afforded. This restriction has to be realized, since serological methods based on an assumed positive correlation between serum antibody level and protection are invalidated by a grave systematic error and are open to severe criticism.

The real correlation is with the level of antibody available at the site of virtual infection, that is, on the respiratory surface. Consequently, the nearest to a single criterion whereby the immunological state of an individual could be judged would be the bronchial antibody level. In all the above experiments and in those to be reported in the following papers, we never failed to find correspondingly larger amounts of free antibody in bronchial washings of groups which later proved to be more resistant to infection than in those which

were less protected. The quantitative data of the Tables appear rather eloquent in this respect, and allow of definite conclusions. Resistance to experimental influenza infection shows a strict positive correlation with the amount of specific anti-haemagglutinin present in the respiratory tract. The bronchial antibody content, and hence the degree of protection, varies independently from the serum antibody level, as it is governed not only by mechanisms at work in the production of antibody but also by factors which influence its distribution. It remains to be seen whether the greatly increased resistance after infection with active virus is wholly due to the fact that the bronchial antibody is approximately 3–10 times that after nasal immunization with killed virus, or whether other factors are also concerned. The nature of these mechanisms has been further investigated, and will be published in later papers of this series.

SUMMARY.

Mice were immunized with graded doses of influenza virus vaccines (live or inactivated by formaldehyde or heat) by three different routes (intranasal, peritoneal or subcutaneous). At the height of their serological response the animals were tested for their resistance to experimental infection with influenza virus type A and B. A new method was devised to assess quantitatively the protection afforded by immunization.

Mild experimental infection gives rise to an approximately 10^9-fold increase in specific resistance to re-infection with the homologous strain.

Peritoneal vaccination with 200–300 agglutinating doses of live, formol- or heat-killed virus increases protection by the order of 1,000, the same amount of virus given subcutaneously by approximately 100.

The resultant immunity is a direct function of the dose of virus given in the vaccine. There is no detectable difference in immunizing power of live or inactivated vaccines of identical haemagglutinin content when administered by one of the extra-respiratory routes.

Given intranasally inactive virus lacking enzyme activity (heated vaccines) provides better protection than an equivalent amount of enzymically active killed vaccine (formolized virus).

None of the vaccines, administered by any of the routes used, affords any protection against infection by the heterologous type of virus.

The results are correlated with the serological findings at the day of challenging, and it is shown that resistance to infection varies independently from the serum antibody titres, but that there exists a strong positive correlation between the antihaemagglutinin content of bronchial washings and the degree of specific immunity. On these grounds the use of the serum antibody level as the measure of susceptibility to infection is criticized, and the necessity of determining the antibody present at the site of possible infection stressed.

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STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

V. ENHANCEMENT OF IMMUNITY BY PATHOTOPIC VACCINATION

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The results presented in papers III and IV showed marked variations in both the serological and immune response of mice to various types of vaccination. The quantity and distribution of antibody varied depending on the site of immunization, and these differences were also fully reflected in the developing resistance of the animals to homologous challenge. It was demonstrated that the degree of protection is determined by the amount of specific antibody in the respiratory tract, and not by that contained in the serum. Consequently, that type of vaccination is more economical and efficient which has a higher "antibody distribution coefficient", that is, results in a higher percentage of the antibody produced being free in the bronchi. Judged by this criterion, the nasal route of immunization appears superior to extra-respiratory ones.

However, while clearly establishing the facts, our experiments gave no information as to the mechanism underlying these differences in antibody distribution. In view of the obvious practical interest of the findings, further experiments were devised to elucidate this side of the question. Since the results could be equally satisfactorily explained by local production or local concentration of antibody (of which we, instinctively, favoured the first alternative), in the decisive test mice were immunized simultaneously with influenza A and B virus. One of the antigens was given by the nasal route, the other peritoneally. If antibody were produced locally, a difference should be detected in the bronchial washings: high titre antibody against the nasally given antigen, and practically none against the peritoneally given one.

The actual experiment gave, to our greatest surprise, the diametrically opposite result. After double immunization it was the antibody against the peritoneally given antigen that appeared in higher concentration in the bronchial washings; the resistance of the respective group against challenge with active virus homologous to the peritoneal vaccine was elevated accordingly. A fortunate accident, this finding not only pointed to local accumulation as the mechanism of antibody distribution after nasal immunization, but also revealed a prac-

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tical means of altering this distribution, and of directing antibody present in the circulation into the respiratory tract.

The phenomenon was studied in detail; this paper gives the results, both serological and protective, of double immunization experiments on mice using influenza virus as antigen.

The previous papers, of which this is a direct continuation, contain detailed descriptions of all *materials and methods* used; a repetition seems superfluous.

EXPERIMENTS.

The experimental technique was of a standard type throughout this study. Groups of 6–7 weeks-old mice were immunized by the nasal, peritoneal or subcutaneous route, as described in Fazekas de St.Groth and Donnelley (1950 a). Eleven days later ten mice of each group were killed and the antibody content of the individual sera and bronchial washings determined. The Tables below give the geometric mean as "average antibody titre" of each group on the day of challenging. Dilutions of active virus (10^0–10^{-5}) were used as infective inoculum, and the "protection test" evaluated by the method previously described (Fazekas de St.Groth and Donnelley, 1950 b). As in paper IV, all Tables give (1) the experimentally determined "total lung lesions" (ΣL) of each group, (2) the calculated difference of this value from that of the control group (d_x), (3) the equivalent of a tenfold reduction in infectivity, as found in normal mice (d_{10}), and (4) the "protection values" (P), characteristic of the immunological state of the respective experimental group. The preparation of the vaccines ("active", "formol", "heated") has also been described. Two classical strains of influenza virus were used: Melbourne (A) and LEE (B). 0·05 ml. volumes were given nasally, and 0·10 ml. by the peritoneal or subcutaneous routes. The haemagglutinin titre of the vaccines is specified in the Tables.

In the *first experiment* formolized vaccines were given by the nasal and peritoneal route. As negative control, formolized normal allantoic fluid was used. The symbols designating the vaccines in the Tables will be: *A* (A-type, strain MEL), *B* (B-type, strain LEE) and *O* (normal allantoic fluid); for the routes of administration: *n* (nasal) and *p* (peritoneal). Thus, e.g. "*AnBp*" means type A (MEL) vaccine given nasally and type B (LEE) vaccine given peritoneally; or, "*OnAp*" stands for normal allantoic fluid given nasally and type A (MEL) given peritoneally. Vaccination was always simultaneous, i.e. the interval between the administration of the two inocula never exceeded three hours.

In order to control the experiment rigidly, all reasonable combinations of the vaccines were used, and all groups were challenged with both types of live virus eleven days after immunization. Tables 1 and 2 give the results in detail.

Analysis of the quantitative data reveals the following facts: (a) heterologous virus or normal allantoic fluid given by any route does not cause significant changes in the resistance of mice to a type of virus they have not been in contact with; (b) the degree of resistance to homologous challenge after vaccination depends on the site of immunization, and is proportional to the antibody content of the respiratory tract. These two points have been established previously (Paper IV), and serve only as a negative control in this experiment. The novel finding is the highly significant increase in both bronchial antibody content and specific resistance when peritoneal immunization is combined with simultaneous nasal administration of an unrelated vaccine which in itself is

TABLE 1.

Protection after single and double vaccination with formolized virus.

Challenging strain: Melbourne (A)

Vaccination	Antibody against MEL		Infectivity test			Protection
	serum	bronchi	ΣL	d_x	log$P$	(P)
An	39	2·3	28·96	7·11	3·50	3,160
AnBp	43	2·1	29·45	6·62	3·26	1,840
Ap	141	1·1	30·83	5·24	2·58	380
ApOn	128	1·2	31·09	5·92	2·91	812
ApBn	124	3·9	27·24	8·83	4·35	22,400
Bn	—	—	35·28	0·79	0·39	2
Bp	—	—	35·93	0·14	0·07	1
On	—	—	36·74	—0·67	—0·35	—2
Nil (control)	—	—	36·07	d_{10} = 2·03	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

Vaccines: *A* = type A (Melbourne strain), titre: 1,000;
B = type B (LEE-strain), titre: 640;
O = normal allantoic fluid + 0·05 p.c. formaldehyde;
n = nasally;
p = peritoneally.

TABLE 2.

Protection after single and double vaccination with formolized virus.

Challenging strain: LEE (B).

Vaccination	Antibody against LEE		Infectivity test			Protection
	serum	bronchi	ΣL	d_x	log$P$	(P)
Bn	31	2·7	24·13	7·50	3·25	1,780
BnAp	24	2·5	24·73	6·90	2·99	977
Bp	140	0·7	27·27	4·36	1·89	78
BpOn	157	0·9	26·61	5·02	2·17	148
BpAn	125	4·9	22·41	9·22	4·00	10,000
An	—	—	30·62	1·01	0·44	3
Ap	—	—	32·25	—0·62	—0·27	—2
On	—	—	30·14	1·49	0·65	4
Nil (control)	—	—	31·63	d_{10} = 2·305	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

A = MEL-vaccine, titre: 1,000;
B = LEE-vaccine, titre: 640.

incapable of affording even the slightest protection. Equally clear with both virus vaccines used, this enhancement of protection is not brought about by the formolized allantoic fluid (the "diluent" of the antigens). This finding, an important lead to the better understanding of the immunization process, will be followed and exploited in a later publication.

That the developing higher protection is not the outcome of the simultaneous presence of two antigens in the animal organism, is borne out by the fact that it is *only* the immunity against the peritoneally given antigen which is enhanced by double vaccination; protection afforded by the nasally administered vaccine is practically identical in animals which received the heterologous strain peritoneally and in those which did not. This finding, taken together with the serological data, shows that enhanced resistance in this case is not due to increased antibody production, but to the different distribution of antibody present in the organism, that is, to a higher percentage free in the bronchi without any obvious change in serum titre.

Further evidence for this point is furnished by the experiments following, all of which are, in a certain sense, only a horizontal expansion of the basic experiment just related.

The results of the *second experiment* performed with heated vaccines in place of formolized, are practically identical with those of the first experiment. Nasal administration of a heterologous virus enhances protection afforded by peritoneal vaccination to a highly significant degree (see Tables 3 and 4). The increase of immunity runs parallel with the shift in antibody distribution: there is a marked rise in bronchial antibody titres without a proportional change in serum titres. Again, the phenomenon is elicited only by the nasal instillation of a virus-containing fluid but not by normal allantoic fluid.

TABLE 3.

Protection after single and double vaccination with heated virus.

Challenging strain: Melbourne (A)

Vaccination	Antibody against MEL		Infectivity test			Protection
	serum	bronchi	ΣL	d_x	log$P$	(P)
An	64	4·3	24·84	9·45	3·96	9,120
AnBp	59	4·1	24·17	10·12	4·20	15,800
Ap	112	0·8	29·38	4·91	2·04	110
ApOn	134	0·9	28·92	5·37	2·23	170
ApBn	98	3·7	25·15	9·14	3·79	6,170
Bn	—	—	32·75	1·54	0·64	4
Bp	—	—	33·86	0·43	0·18	2
Nil (control)	—	—	34·29	d_{10} = 2·41	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

A = MEL-vaccine, titre: 520;

B = LEE-vaccine, titre: 300.

TABLE 4.

Protection after single and double vaccination with heated virus.
Challenging strain: LEE (B).

Vaccination	Antibody against LEE		Infectivity test			Protection
	serum	bronchi	ΣL	d_x	log$P$	(P)
Bn	24	2·3	25·69	6·60	2·81	645
BnAp	31	2·1	26·38	5·91	2·53	339
Bp	98	—	28·97	3·32	1·42	26
BpOn	76	0·6	27·92	4·37	1·87	74
BpAn	84	1·8	25·11	7·18	3·07	1,170
An	—	—	32·55	—0·26	—0·11	1
Ap	—	—	31·33	0·96	0·41	3
Nil (control)	—	—	32·29	d_{10} = 2·34	0·00	1

— = <5 in serum and <0·5 in bronchial washings.
A = MEL-vaccine, titre: 520;
B = LEE-vaccine, titre: 300.

With small quantitative differences, identical results were obtained in the *third experiment* in which active virus was given by the peritoneal and subcutaneous routes, and a heterologous formol-vaccine nasally. As in the above experiments, the "nasal adjuvant" caused a 50–500-fold increase in specific resistance with an associated rise in bronchial antibody concentration. The fact that immunity following either peritoneal or subcutaneous vaccination could be enhanced by nasal administration of a non-protective heterologous vaccine, is sufficient to demonstrate the general character of the phenomenon.

Correlation Between Immunizing Dose and Protection.

If the "nasal adjuvants" acted by directing the antibody produced against the homologous vaccine given by some other route, that is by increasing the antibody distribution coefficient in favour of the bronchi, the enhanced protection should be a direct function of the serum antibody titres. In order to test the validity of this hypothesis, the following experiment was performed.

Groups of mice were immunized peritoneally with different amounts of formolized MEL (A) vaccine. As shown in the previous papers (Fazekas de St.Groth and Donnelley, 1950 a, b), while both antibody titres and specific resistance are proportional to the immunizing dose, the antibody distribution coefficient (ratio of bronchial and serum titres) remains constant throughout the range of dilutions tested. Half of the mice in each group received, simultaneously with the peritoneal injection, an intranasal inoculum of the heterologous LEE (B) virus. All groups were challenged eleven days after immunization with active Melbourne (A) virus. Table 5 gives the results of the protection test, together with the serological data (serum and bronchial antibody titres) on the day of challenging.

TABLE 5.

Correlation between immunizing dose and protection.

Formolized vaccines. Challenging strain: Melbourne (A).

Vaccination perit. (MEL)	Vaccination nasal (LEE)	Antibody against MEL serum	Antibody against MEL bronchi	Infectivity test ΣL	Infectivity test d_x	Infectivity test log$P$	Protection (P)
	nil	167	1·1	31·14	5·29	2·19	155
10^0	10^0	211	2·9	26·84	9·59	3·98	9,550
	nil	124	0·7	31·98	4·44	1·84	69
$10^{-0.5}$	10^0	102	2·1	28·17	8·26	3·43	2,690
	nil	42	—	32·76	3·67	1·52	33
$10^{-1.0}$	10^0	50	1·4	30·31	6·08	2·52	331
	nil	17	—	34·23	1·80	0·75	6
$10^{-1.5}$	10^0	27	0·9	31·42	5·01	2·08	120
	nil	12	—	35·80	0·63	0·26	2
$10^{-2.0}$	10^0	9	—	33·02	3·41	1·42	26
nil	nil	—	—	36·43	$d_{10}=$ 2·41	0·00	1

— = <5 in serum and <0·5 in bronchial washings.
MEL-vaccine titre: 960;
LEE-vaccine titre: 640.

The experimental findings strongly support our working hypothesis. The gradation in the immune response to graded doses of vaccine is evident, as is the gradation in the enhancing effect of the "nasal adjuvant". In no instance was there any significant change in the serum antibody level, while the bronchial titres rose in every case, proportionally to the serum titres. The shift in antibody distribution was followed by a marked rise in specific resistance. That this enhancement is a function of the original immunity, is borne out by the protection values of "adjuvated" and "non-adjuvated" groups which—within the limits of experimental error—give a fairly constant ratio over the range of hundredfold differences in immunizing dose.

Relationship Between Route of Vaccination and Adjuvanation.

All the above findings suggest that the enhancing effect of a "nasal adjuvant" is due to its changing the distribution of specific antibody present in the animal body, so as to attain a relatively higher concentration in the respiratory tract. The following experiment furnishes formal proof for two corollaries of this assumption: (1) a "nasal adjuvant" should not enhance protection afforded by a nasally given vaccine, and (2) heterologous vaccine given by any route but the nasal, should not enhance specific resistance.

Formolized Melbourne (A) virus was used as immunizing vaccine and formolized LEE (B) virus, the heterologous type, as adjuvant. All groups were challenged with dilutions of active MEL (A) virus. Table 6 shows the antibody titres on the day of challenging (11 days after vaccination) and the results of the protection tests.

TABLE 6.

Relationship between route of vaccination and enhancement of protection.

Challenging strain: Melbourne (A).

Vaccination	Antibody against MEL		Infectivity test			Protection
	serum	bronchi	ΣL	d_x	log$P$	(P)
An	47	4·1	24·31	10·31	4·51	32,400
AnBn	61	4·9	25·45	9·17	4·01	10,200
AnBp	54	3·7	26·09	8·51	3·72	5,250
AnBs	41	4·1	24·76	9·92	4·32	20,900
Ap	189	1·8	28·94	5·68	2·48	302
ApBn	154	3·2	25·11	9·51	4·16	14,500
ApBp	207	1·7	30·07	4·55	1·99	98
ApBs	216	2·1	28·35	6·27	2·74	550
As	74	0·6	31·16	3·42	1·49	31
AsBn	49	2·1	27·84	6·78	2·96	910
AsBp	60	—	31·94	2·68	1·17	15
AsBs	82	0·5	32·63	1·99	0·87	7
Nil (control)	—	—	34·62	d_{10} = 2·29	0·00	1

— = <5 in serum and <0·5 in bronchial washings.

Vaccines: *A* = type A (Melbourne strain), formolized; titre: 1,200
B = type B (LEE strain), formolized; titre: 720
n = nasally
p = peritoneally
s = subcutaneously

Immunity after peritoneal or subcutaneous vaccination is enhanced by simultaneous nasal administration of a heterologous vaccine, concomitantly with an increase in the bronchial antibody content. The efficiency of nasal vaccination is not enhanced by a nasal adjuvant (*AnBn*), neither is the distribution coefficient of the anti-A immune-body changed by this procedure. Further, B-virus given peritoneally or subcutaneously does not enhance resistance afforded by A-vaccines given by any of the three routes used in our experiments; similarly, there is no detectable change in antibody distribution after pararespiratory administration of a heterologous vaccine.

DISCUSSION.

The experimental evidence presented in this study shows that specific resistance after immunization by extra-respiratory routes with influenza virus vaccines can be significantly enhanced by simultaneous nasal administration of a heterolo-

gous virus, in itself incapable of affording any protection. To distinguish it from the enhancement of immunization by local (isotopic) use of adjuvants admixed with the vaccines, the phenomenon will be termed *pathotopic potentiation*. Any substance capable of eliciting such a response will be called a *pathotopic adjuvant*, an agent effective only at the appropriate site, viz. at the site of possible infection.

The increased resistance can be directly attributed to the higher concentration of antibody present in the respiratory tract, since the application of a nasal adjuvant invariably resulted in a change of antibody distribution with higher titres in bronchial washings without any noticeable change in the serum level. The latter is not surprising in view of the quantitative data, as the 300–600 p.c. increase in bronchial titres would mean only 1–3 p.c. decrease in serum titres, a difference that escapes detection by the available methods.

It was shown that the phenomenon is independent of the amount of circulating antibody: irrespective of the serum titres a 20–100-fold increase in protection is brought about by the use of pathotopic adjuvants. Further, the type of vaccine used has no influence on the final result either: similar enhancement was found after immunization with active, formolized or heated virus.

However, if specific immunity is induced by nasal vaccination which itself has a high antibody distribution coefficient, the simultaneous administration of heterologous virus has no enhancing effect. This finding taken together with the fact that heterologous virus given by any other route than the nasal does not act as adjuvant, proves (a) that the phenomenon is not due to the simultaneous presence of two vaccines in the organism, and (b) that the limiting, highest distribution of antibody is that found after nasal immunization with either active or killed vaccines.

Analysis of the quantitative serological results shows that pathotopic potentiation is fundamentally different from the enhancement of immunity by use of local adjuvants capable of increasing the serological response and resulting in a prolonged persistence of high antibody titres. In our experiments the absolute amount of antibody produced remained unchanged, as the nasal adjuvant exerted its effect only on the *distribution* of antibody, a fact well expressed in the consistent rise in the distribution coefficients, i.e. in the ratio bronchial antibody/serum antibody. In other words, the pathotopic agent does not act by reinforcing antibody production but by the strategic use of antibody already present. This statement implies that any type of immunity, active or passive, in which the antibody distribution coefficient is lower than after nasal vaccination (<5) should be enhanced by pathotopic potentiation; further, that any substance capable of shifting the distribution of antibody in favour of the respiratory surface should act as adjuvant in the case of influenza. Presentation of experimental evidence concerning this point will be the subject of later papers of this series, and the fuller discussion of the underlying mechanisms is deferred till then.

SUMMARY.

Specific resistance after immunization by extra-respiratory routes with influenza virus vaccines can be significantly enhanced by simultaneous nasal administration of a heterologous virus, in itself incapable of affording any protection. The phenomenon is termed *pathotopic potentiation*, and any substance capable of eliciting scuh a response, a *pathotopic adjuvant*.

The increased resistance is positively correlated with an increase in the specific anti-haemagglutinin content of bronchial washings; there is no detectable change in the serum antibody level, i.e. the enhancement is brought about by a change in the *distribution* and not in the quantity of antibody produced.

Irrespective of its degree, the initial immunity resulting from extra-respiratory vaccination was increased by 20–100-fold, with a corresponding shift in the antibody distribution coefficient.

It is demonstrated that the phenomenon is not due to the simultaneous presence of two antigens in the organism, and that only states of immunity with a low antibody distribution coefficient can be potentiated, and these only by the administration of the adjuvant by the appropriate route (at the site of virtual infection).

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A TECHNIQUE FOR THE ASSAY OF SMALL QUANTITIES OF INSULIN USING ALLOXAN DIABETIC, HYPOPHYSECTOMIZED, ADRENALECTOMIZED RATS

by J. BORNSTEIN[1]

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The aim of this experiment was to produce an animal preparation sufficiently sensitive to assay insulin concentration in human plasma, so that a study of such concentrations could be made in normal and diabetic subjects.

Using hypophysectomized, adrenomedullated rats, Gellhorn and Feldman (1941) were able to determine insulin concentrations in the dog. More recently Anderson *et al.* (1947), described a technique using adrenomedullated, alloxan diabetic, hypophysectomized rats.

Houssay and Magenta (1925) showed that hypophysectomy markedly increased sensitivity to insulin. Cannon, McIver and Bliss (1924) demonstrated that adrenaline is secreted in response to insulin hypoglycaemia, and Hemmingsen, Nielsen and Nielsen (1938) showed that adrenalectomized mice have a marked hypersensitivity to insulin.

Therefore, for the purpose of the present study it appeared that a hypophysectomized, adrenalectomized animal would be the most suitable preparation. However, such preparations were found to have a very short life, for they were extremely liable to spontaneous hypoglycaemia. Also surviving animals had blood sugar concentrations very close to hypoglycaemic levels. When they were given minute doses of insulin fatal hypoglycaemia frequently occurred.

For the above reason, and because it was considered desirable to remove possible interference from endogenous insulin secretion, it was decided to use an alloxan diabetic, hypophysectomized, adrenalectomized rat (A.D.H.A.).

EXPERIMENTAL.

Method of Preparation of the A.D.H.A. Rat.

The following technique was found to have the lowest overall mortality:
Adult male rats of approximately 220–240 gm. were taken from stock.

[1] Working under a full-time grant from the National Health and Medical Research Council.

Diabetes was produced by the intravenous injection of alloxan (50 mg./kg.) into a tail vein. Thirty-six hours later, the animals were checked for glycosuria and ketonuria and, if showing severe glycosuria and ketonuria, or having a blood sugar of over 350 mg. p.c. were then given 4 units of P.Z. insulin daily for four days.

After a period of 48 hours without insulin administration, hypophysectomy was carried out by a technique adapted from Thompson (1932), using open ether anaesthesia.

Unless this interval of 48 hours was given, there was a considerable mortality at this stage due to hypoglycaemia. As a further precaution against hypoglycaemia, the animals were given 0·25 gm. of glucose intraperitoneally prior to operation.

After the hypophysectomy wounds had healed, the adrenals were removed through a single midline incision in the back.

The animals were ready for use as soon as the adrenalectomy wounds had healed.

The mortality of the entire procedure in our hands has been about 1 in 3.

The total time of preparation was approximately 25 days.

The A.D.H.A. rat prepared in this manner weighs 180± 10 gm. and usually has a blood sugar concentration of 120–160 mg./100 ml. Immediately after the feeding of carbohydrates the blood sugar may rise to the vicinity of 300 mg. but settles to the usual level within one hour.

Rats so treated are very sensitive to food lack. Periods of six hours starvation have at times been sufficient to produce fatal hypoglycaemic reactions.

They are also extremely sensitive to cold and will die if exposed to temperatures of the order of 10–12° C.

Kept under good conditions with temperatures of 22–26° C., and given unlimited food, the A.D.H.A. animals live for up to eight months, and can be used for up to 24 assays during this time.

Technique of Assay.

It was decided to give insulin subcutaneously for two reasons.

In the first place anaesthesia, which influences carbohydrate metabolism, is avoided.

Secondly, the effect of a given subcutaneous dose of insulin, although slower in development, is more profound than when it is administered intravenously.

Preliminary experiments showed that, when the animals were allowed to feed up to the time of use and then incubated for three hours, the blood sugar was unstable for the first hour, stable in the second hour, and then tended to fall (Table 1).

TABLE 1.

Blood sugar concentrations in mg./100 ml. of 8 A.D.H.A. rats kept in an incubator at 37° C. for three hours.

Rat	Start	1 hour	2 hours	3 hours
1	168	162	160	154
2	140	130	133	128
3	174	147	145	139
4	158	159	157	158
5	196	152	154	148
6	122	118	120	116
7	161	141	143	138
8	153	122	123	118

From Table 1 it is seen that the animals required a period of one hour to allow the blood sugar to become stabilized. The observations then had to be made over the next hour, as beyond that time the insulin effect became confused by the normal fall in blood sugar.

Therefore, the following technique was used throughout:

1. Groups of 8 animals were used at each assay, as larger numbers led to great delays in handling.
2. The animals were placed in individual cages, to which water but no food was supplied. They were then placed in the incubator at 37° C. for one hour.
3. The animals were then removed and blood taken for sugar estimations from tail vein.
4. The animals were then rapidly injected subcutaneously with the test solution. (If the handling is rapid, there is little or no struggling, and stages 3 and 4 were completed in twelve minutes.)
5. The animals were then returned to the incubator for one hour.
6. The animals were then removed and blood taken from the tail veins for sugar estimations.
7. The animals were then returned to a common cage and fed.

It has been found that the best results are obtained if the group is permitted to have from four to seven days' rest before being used again.

In the writer's experience, if, during the assay procedure, an A.D.H.A. rat develops a hypoglycaemic reaction it is inevitably fatal. All measures used to raise the blood sugar are without effect. Such reactions usually appear at blood sugar concentrations of about 60 mg./100 ml.

Results.

Results are expressed in Table 2.

The amount of insulin used is shown in milliunits. (1 milliunit = 0·001 unit of insulin.)

TABLE 2.

Amount of insulin in milliunits		Number of A.D.H.A. rats used for estimation	Mean depression of blood sugar mg./100 ml.	Standard deviation	Standard error of mean
a.	0·5	23	63·7	11·60	2·44
b.	0·25	17	49·5	8·94	2·24
c.	0·2	39	44·5	9·90	1·75
d.	0·13	18	34·8	7·07	1·62
e.	0·10	44	28·5	7·75	1·17
f.	0·08	15	23·3	8·00	2·05
g.	0·05	18	16·5	12·43	3·11

The individual results, means, and relation of the fall in blood sugar concentration to the amount of insulin injected are shown in Fig. 1.

DISCUSSION.

Analysis of the results given in Fig. 1 shows a straight line relationship between logarithms of milliunits of insulin and depression of blood sugar.

Since the effect may thus be represented by a straight line, it is not necessary to establish significant differences between the various means. The trend having been once established, it may be inferred that a change in insulin dosage will result in a change in the mean depression of blood sugar. The equation relating depression to logarithm of insulin in milliunits is $D = 78{\cdot}5 + 49{\cdot}1\ L$. The standard error of the regression coefficient is 2·4.

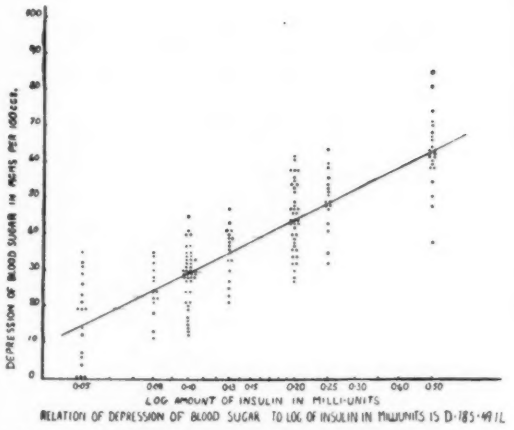

Fig. 1. A technique for the assay of small quantities of insulin using Alloxan diabetic, hypophysectomized, adrenalectomized rats.

The observed and calculated depressions are shown in Table 3.

TABLE 3.

Amount of insulin in milliunits	Mean depression of blood sugar	Calculated mean depression of blood sugar
0·50	63·7	63·7
0·25	49·5	48·9
0·20	44·5	44·2
0·13	34·8	35·0
0·10	28·5	29·4
0·08	23·3	24·6
0·05	16·5	14·6

The direct relationship between the logarithm of the dose of insulin and the depression in blood sugar, found by Anderson *et al.* (1947), has been confirmed and is shown to extend down to 0·05 milliunit; in view of the wide scatter at the lower end of the curve, it is considered that 0·05 milliunit represents the lower limit of insulin which can be estimated by this method. The method is more sensitive than those previously described.

SUMMARY.

A method of using alloxan diabetic, hypophysectomized, adrenalectomized (A.D.H.A.), rats for the assay of small quantities of insulin is described.

A technique for the preparation of A.D.H.A. rats is described.

The range of the method is between 0·50 milliunit and 0·05 milliunit of crystalline insulin.

The statistical significance of the results obtained is demonstrated.

Acknowledgment. I would like to thank Mr. E. J. Williams of the Section of Mathematical Statistics, C.S.I.R.O., for performing the statistical analysis.

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NORMAL INSULIN CONCENTRATION IN MAN

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As far as the author is aware, there is no recognized standard method for the measurement of blood insulin concentrations using only 10 ml. or less of blood.

In view of this, the technique described in a previous paper (Bornstein, 1949) was applied to this problem.

Six subjects, either normal in all respects or suffering from chronic conditions not affecting carbohydrate metabolism, were used for this study, which was carried out under the conditions of a glucose tolerance test.

EXPERIMENTAL.

10 ml. of blood were taken and put into a centrifuge tube containing 1 ml. of 3 p.c. sodium citrate, and fresh plasma prepared. By this technique the depression of blood sugar was measured in a group of alloxan diabetic, hypophysectomized, adrenalectomized (A.D.H.A.) rats under the standard conditions previously described.

0·5 ml. of plasma was used as the test amount, for previous experience with the A.D.H.A. rat, and the results obtained by Gellhorn *et al.* (1941) in the dog suggested that 1 ml. would lead to large losses of animals from hypoglycaemic reactions.

Although this technique assays plasma insulin concentration, it is considered by the writer that this concentration does not necessarily reflect exactly the secretion of insulin by the pancreatic islets, because a certain amount must be immediately utilized.

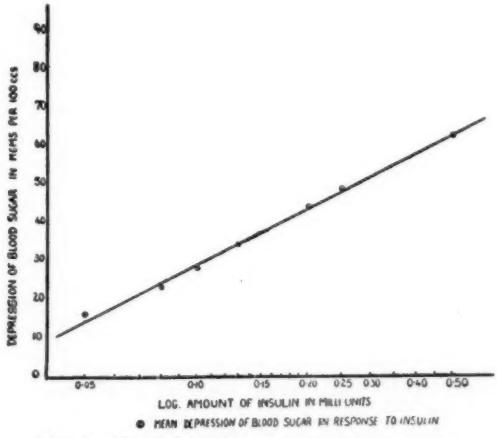

Fig. 1. Normal insulin concentration in man.

Results.

The results obtained are presented in the following Tables, which show the depression of blood sugar in mg. per 100 ml. in A.D.H.A. animals following the injection of 0·5 ml. plasma obtained from the subjects indicated.

[1] Working under a full-time grant from the National Health and Medical Research Council, Australia.

It is to be noted in each Table the first two columns of readings (1—2, 5—6, 9—10, 13—14) are obtained following injection of plasma obtained from the same subject (S.B.).

It is to be stressed that the groups of eight rats are numbered purely for convenience and do not numerically correspond to each other.

Fig. 1, which is taken from the data of a previous paper (Bornstein, 1949), shows the relationship between the depression in blood sugar and the logarithm of the amount of insulin injected.

TABLE 1.

Depression of blood glucose concentration observed in A.D.H.A. rats following the injection of 0·5 ml. plasma subcutaneously.

Fasting plasma.				
Assay	1	2	3	4
Subject	S.B.	S.B.	J.W.	B.E.
Subject's F.B.S.	98 mg. %	90 mg. %	104 mg. %	96 mg. %
Rat	Depression of blood glucose concentration in mg./100 ml.			
1	6	6	7	8
2	6	9	9	11
3	11	14	16	16
4	14	14	19	18
5	17	19	22	21
6	19	23	27	29
7	24	26	30	31
8	27	31	34	35
Mean	15·5	18	20·5	21
Standard deviation	7·76	8·58	9·66	9·73
Overall mean		18·7		

As for assays 1 and 4, T is 1·25, P is 0·3, there is no statistically significant difference between any of the above means.

The overall mean corresponds to an absolute amount of 0·05 milliunit of insulin, therefore the fasting plasma insulin concentration is 0·1 milliunit of insulin per ml. of plasma.

TABLE 2.

Plasma 1 hour after ingestion of glucose.

Assay	5	6	7	8
Subject	S.B.	S.B.	M.J.	J.K.
Subject's B.S.	154 mg. %	146 mg. %	152 mg. %	163 mg. %
Rat	Depression of blood sugar in mg. per 100 ml.			
1	14	18	12	14
2	20	27	19	18
3	23	27	27	29
4	26	29	31	32
5	29	33	32	34
6	31	35	36	36
7	35	38	38	38
8	39	40	38	41
Mean	27	31	29	30
Standard deviation	8·12	5·07	9·38	9·58
Overall mean		29·3		

As for assays 5 and 6, T is 1·17, P is 0·3, there is no statistically significant difference between any of these.

The overall mean corresponds to an absolute amount of 0·10 milliunit of insulin, therefore the plasma insulin concentration one hour after the ingestion of glucose is 0·20 milliunit per ml. of plasma.

TABLE 3.

Plasma 2½ hours after the ingestion of glucose.

Assay	9	10	11	12
Subject	S.B.	S.B.	M.J.	J.W.
Subject's B.S.	104 mg. %	98 mg. %	102 mg. %	96 mg. %
Rat	Depression of blood sugar concentration in mg. per 100 ml.			
1	29	29	26	24
2	34	32	31	30
3	37	35	34	33
4	39	38	37	38
5	43	40	41	39
6	44	43	44	41
7	48	47	48	43
8	52	51	49	46
Mean	41	39	38	37
Standard deviation	7·52	7·47	8·23	7·29
Overall mean	38·9			

As for assays 9 and 12, T is 1·08, P is 0·3, there is no statistically significant difference between any of the above means.

The overall mean corresponds to an absolute amount of 0·17 milliunit of insulin, therefore the plasma insulin concentration 2½ hours after glucose is 0·34 milliunit per ml. of plasma.

TABLE 4.

Plasma 3½ hours after the ingestion of glucose.

Assay	13	14	15	16
Subject	S.B.	S.B.	B.E.	A.S.
Subject's B.S.	104 mg. %	98 mg. %	102 mg. %	96 mg. %
Rat	Depression of blood sugar in mg. per 100 ml.			
1	14	12	17	19
2	16	24	24	23
3	21	27	28	29
4	28	29	31	33
5	29	33	34	36
6	35	38	39	41
7	39	39	43	43
8	44	46	46	47
Mean	28	31	33	34
Standard deviation	10·1	10·5	9·35	9·82
Overall mean	31·5			

As for assays 13 and 16, T is 1·21, P is 0·3, there is no statistically significant difference between any of the above means.

The overall mean corresponds to an absolute amount of 0·11 milliunit of insulin, therefore the plasma insulin concentration 3½ hours after the ingestion of glucose is 0·22 milliunit per ml. plasma.

The relationship of the plasma insulin level to the blood sugar level is shown in Fig. 2.

DISCUSSION.

Analysis of the data from Tables 1, 2, 3 and 4 shows that in no case was there significant difference between patients, and it is concluded that the results for different patients at any time can be validly combined. Moreover, the variances for each patient at each time are sufficiently homogeneous for them to be combined to give a single estimate of variability in the data.

The means and the differences required for significance are as follow:—

TABLE 5.

	Depression in blood sugar
Fasting plasma	18·7 mg. %
1 hour after ingestion of glucose	29·3 mg. %
2½ hours after ingestion of glucose	38·9 mg. %
3½ hours after ingestion of glucose	31·5 mg. %

Significant difference between two means: 5 p.c. level 4·3; 1 p.c. level 5·7.

Thus it is seen that all results show differences significant at the 1 p.c. level between the four Tables.

It is also seen from the above that the curve shown for plasma insulin in Fig. 2 represents the rise and fall of plasma insulin following the ingestion of 50 gm. of glucose.

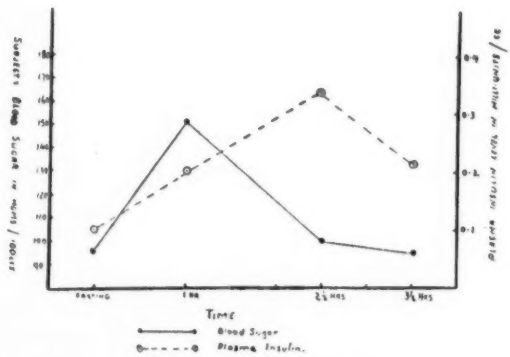

Fig. 2. Normal insulin concentration in man.

A study of Fig. 2 shows that plasma insulin concentration increases as the blood sugar rises, reaching a maximum one hour after the peak of the blood sugar curve, and thereafter declines. The further fall in blood sugar which then occurs, and which is frequently seen in standard blood sugar curves, is considered to be due to the excess of insulin present after the blood sugar has returned to normal. This excess is shown to be present 3½ hours after glucose has been ingested.

Anderson (1947) showed that, in the isolated pancreas, insulin was secreted when the glucose content of the perfusing fluid was increased. This finding is indirectly confirmed by the present experiment.

SUMMARY.

Plasma insulin concentrations in man have been measured under the conditions of a glucose tolerance curve, using alloxan diabetic, hypophysectomized, adrenalectomized animals.

Under the conditions of the experiment the plasma insulin concentrations were 0·1 milliunit per ml. fasting, 0·2 milliunit per ml. 1 hour after the ingestion of glucose, 0·34 milliunit per ml. 2½ hours after glucose, and 0·22 milliunit per ml. 3½ hours after glucose.

Acknowledgment. I would like to thank Mr. E. J. Williams of the Section of Mathematical Statistics, C.S.I.R.O., for the statistical analysis.

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BIOLOGICAL PROPERTIES OF POLYSACCHARIDE AND LIPOID FRACTIONS FROM A PATHOGENIC STRAIN OF *ASPERGILLUS FUMIGATUS*

by NEVILLE F. STANLEY

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(Accepted for publication 16th September, 1949.)

Aspergillus fumigatus may cause fatal infections in mammals and birds. In man, however, aspergillosis is a rare disease. According to Cooper (1946), cases of pulmonary aspergillosis are clinically and roentgenologically indistinguishable from chronic pulmonary tuberculosis. Natural infections of birds and experimental infections of laboratory animals are characterized by extensive lesions which in the later stages of the disease resemble the tubercles caused by infection with *Myco. tuberculosis*. Little is known concerning the pathogenesis of the disease or the mechanism of tubercle formation by aspergilli. The following investigations were carried out to determine the susceptibility of laboratory animals to infection, the rôle of a lipoid fraction in tubercle formation and the immunological properties of a polysaccharide fraction.

Methods and Materials.

Media.

Modified Czapek-Dox medium: $NaNO_3$, 3·5 gm.; KH_2PO_4, 1·5 gm.; KCl, 0·5 gm.; $MgSO_4 \cdot 7H_2O$, 0·5 gm.; $FeSO_4 \cdot 7H_2O$, 0·015 gm.; glucose, 20·0 gm.; H_2O, 1,000 ml. Autoclave at 10 lb. pressure for 20 minutes.

Modified Sabouraud's medium: glucose, 4·0 gm.; peptone, 1·0 gm.; agar, 2·0 gm.; H_2O, 100 ml. Autoclave at 10 lb. pressure for 20 minutes.

Isolation of the Organism.

The strain of *Aspergillus fumigatus* used in these experiments was isolated, at *post-mortem* examination on 31/3/49 from tubercles occurring in a *Gentu* penguin which died from aspergillosis soon after importation from Antarctica. Post-mortem examination of the penguin revealed numerous firm tubercles on the trachea, bronchi, lungs, crop, pericardium, pleurae and peritoneum. The liver, spleen, kidney and lower portion of the peritoneum remained unaffected. A few small tubercles were scattered in the omentum. The tubercles varied in size from 1 to 5 mm. in diameter. The larger tubercles were disc-shaped and some of them had a grey-green centre. To facilitate the microscopic demonstration of mycelia in the tubercles it was necessary to digest them in N/NaOH for 18 hours. The material could then be squashed between two slides and examined directly or by fixing and staining with 1 p.c. aqueous methylene blue. The fungus was isolated in pure culture on Sabouraud's medium from all lesions examined. At 37° C. the growth was prolific; at 22° C. the mould grew more slowly and colonies could only be distinguished macroscopically after 48 hours' incubation.

Spore suspensions used for inoculating animals and media were obtained by gently rubbing, with a loop, the surface growth of a four day culture on Sabouraud's agar in the presence of 5 ml. of sterile normal saline. The suspension was removed, shaken, and used within one hour.

EXPERIMENTAL.

Preparation of Mycelial Extract and Isolation of Polysaccharide and Lipoid Fractions.

Czapek-Dox medium was dispensed in 3 litre quantities in 5 litre Ehrlenmeyer flasks and continually aerated with oxygen. The medium was inoculated with 2 ml. of a spore suspension and then incubated at 37° C. for four days. The growth was prolific and consisted of a mass of white fluffy coherent colonies. Microscopic examination revealed that the growth was composed almost entirely of mycelia. The mycelial mass was filtered off through lint, washed throughly with distilled water, minced in the Waring blendor and treated as illustrated in the schema on p. 101.

Microscopic examination revealed that grinding with sand reduced the long branching mycelia to small fragments not more than 10 μ in length. The saline extract of these disintegrated mycelia will be referred to as the mycelial extract. It gave positive tests for protein and polysaccharide and contained lipoid. It is probably similar to the "cell sap" described by Henrici (1939).

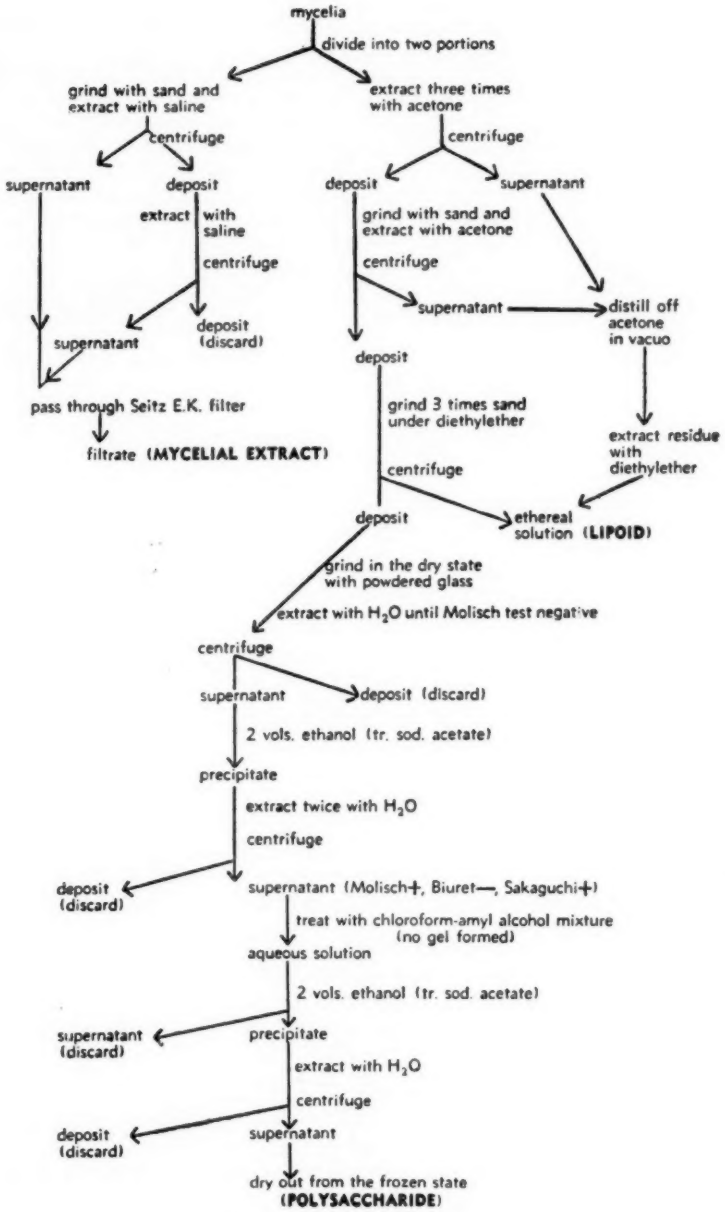
The polysaccharide appeared as a white powder, extremely soluble in water and giving an opalescent solution. It gave a strong Molisch reaction in high dilution. The biuret test was negative, even before extraction with chloroform, but the Sakaguchi test was faintly positive. Bial's test (as modified by Mejbaum) was positive while the Dische cysteine-sulphuric acid test for desoxyribonucleic acid was negative. Tests for nitrogen, sulphur and halogen were negative.

Lipoid material was used for animal inoculation in the form of a saline suspension. This was brought about by evaporating off the ether from the ethereal solution in the presence of normal saline containing 0·03 p.c. sodium lauryl sulphate. The dense suspension formed after the removal of the ether was always shaken well before use and arranged to contain 10 mg. of lipoid per ml. of saline.

Infection of Laboratory Animals with Spore Suspensions of Aspergillus Fumigatus.

Spores were inoculated into the drinking water and the external nares of the test animals. Such treatment caused no demonstrable change in the condition of 3 rabbits, 3 pigeons, 12 guinea-pigs and 12 mice which were observed over a 3 month period. It will be shown later that the differential leucocyte count may serve as a guide in determining whether or not an animal has become infected; but the blood picture of the animals used in the above experiment showed no significant alteration. *Post-mortem* examination revealed no evidence of tubercle formation.

When spores were inoculated by the intraperitoneal route, it was found that the animal readily became infected although in only a few cases did the infection prove to be fatal. In the following experiment eighteen guinea-pigs were inoculated by the intraperitoneal route with 0·5 ml. of a spore suspension. Three guinea-pigs died—one on the fifth day and two on the seventh day after inoculation. Twenty-four hours prior to death the guinea-pigs became slightly emaciated and ataxic. A few hours prior to death ataxia became prominent. The pigs, if not stationary, struggled on their sides, rolled over and over, or moved around in circles.


mycelia
divide into two portions
grind with sand and extract with saline
extract three times with acetone
centrifuge
centrifuge
supernatant
deposit
deposit
supernatant
extract with saline
grind with sand and extract with acetone
centrifuge
centrifuge
supernatant
distill off acetone in vacuo
deposit (discard)
supernatant
deposit
pass through Seitz E.K. filter
grind 3 times sand under diethylether
extract residue with diethylether
filtrate (MYCELIAL EXTRACT)
centrifuge
deposit
ethereal solution (LIPOID)
grind in the dry state with powdered glass
extract with H_2O until Molisch test negative
centrifuge
supernatant
deposit (discard)
2 vols. ethanol (tr. sod. acetate)
precipitate
extract twice with H_2O
centrifuge
deposit (discard)
supernatant (Molisch+, Biuret—, Sakaguchi+)
treat with chloroform-amyl alcohol mixture (no gel formed)
aqueous solution
2 vols. ethanol (tr. sod. acetate)
supernatant (discard)
precipitate
extract with H_2O
centrifuge
deposit (discard)
supernatant
dry out from the frozen state
(POLYSACCHARIDE)

During the course of this experiment skin tests were carried out with the polysaccharide by injecting (intradermally) 0·1 ml. of a saline solution containing 10 mg./ml. The response to intradermal injections of polysaccharide is described later.

To determine the monocytic response differential leucocyte counts were also carried out at frequent intervals. Eventually, *post-mortem* examinations were carried out on all animals and the findings are recorded in an abbreviated form in Table 1. Another batch of pigs was observed over a three month period following intraperitoneal inoculation of spores. Those pigs not dying within 3 weeks did not appear to be sick and survived the observation period.

TABLE 1.

Pig number	Days after injection: animal died on	Days after injection: animal well and killed on	Presence of tubercles	Presence of adhesions	Intradermal test with polysaccharide	Monocytosis
1	5		+	—	*	*
2	7		+	+	*	+
3	7		+	+	*	+
4		10	+	+	*	*
5		13	+	—	+	*
6		17	+	—	+	+
7		20	+	+	+	*
8		24	+	+	+	*
9		27	+	—	+	*
10		30	+	+	*	+
11		31	+	—	+	+
12		34	+	—	+	*
13		40	—	+	+	*
14		42	+	+	*	+
15		42	—	—	*	*
16		42	+	+	+	*
17		42	—	+	+	*
18		42	+	—	*	*

* = test not performed.

The outstanding features of the disease were:

(1) Ataxia prior to death.
(2) The presence of tubercles in the liver, the omentum, the diaphragm, the peritoneal wall and the spleen.
(3) Adhesions between the liver and omentum, omentum and peritoneal wall, or liver and peritoneal wall.
(4) Increase in the number of circulating monocytes.
(5) The isolation, in pure culture, of *Aspergillus fumigatus* from tubercles in every case.

Tubercles were present in the lungs in only one case—that of the guinea-pig dying on the fifth day. Five passages of this strain of *Aspergillus fumigatus* by intraperitoneal inoculation of guinea-pigs did not appear to enhance its virulence.

The lesions in the peritoneal cavity of the guinea-pig resembled (macroscopically and microscopically) the tubercles found in the naturally infected penguin.

Spores were demonstrable in only one case in the tubercles of a guinea-pig which was killed twenty-seven days after infection. In the early stages of the disease, both in birds and guinea-pigs, sections stained with haematoxylin-eosin revealed the tubercle as a mass of

mycelia partly obscured and surrounded by inflammatory cells, mainly polymorphonuclear leucocytes. In some tubercles, necrosis was indicated in the centre of the lesion by multinuclear fragments. In most cases, a small amount of fibro-cellular tissue could be seen surrounding the lesion. No giant cells were seen. Sections of tubercles present twelve days or longer after infection revealed an increase in the fibro-cellular tissue. They were also characterized by the presence of epithelioid and giant cells at the edge of the inflammatory area.

The intravenous inoculation of spores produced a slightly different picture. The disease was invariably fatal after the inoculation of 0·1 ml. of a spore suspension. One pigeon, 3 rabbits, 2 guinea-pigs and 6 mice were inoculated intravenously. The pigeon died on the seventh day, and *post-mortem* examination revealed exceedingly numerous tubercles in the liver and a few in the lungs. The guinea-pigs and mice appeared to have a generalized infection, tubercles being demonstrated in the lung and liver of all animals. The guinea-pigs died on the third day after inoculation and all mice, but one, were dead by the fifth day. The remaining mouse survived 10 days before dying, and three days prior to death exhibited the ataxic phenomenon which was shown by animals if they were observed shortly before death.

The results of intravenous inoculation of spores into rabbits resulted in marked ataxia shortly before death. In one animal the head was twisted 30° to the right. The right eye was focussed downwards and the left eye with definite nystagmus was focussed up and back towards the left ear. Movement of the limbs resulted in the rabbit falling on its right side, from which position it rolled over repeatedly until stopped. *Post-mortem* examination revealed extensive tubercles in the lung, liver, spleen, kidney and omentum. The macroscopic and microscopic appearances of the brain were normal. The middle ear was not examined.

The Haemopoietic Response.

(a) With Infection.

In the rabbit and the guinea-pig this was characterized by a neutrophil leucocytosis and a monocytosis. The actual counts on one of the three rabbits receiving 0·1 ml. of a spore suspension is given in Table 2. One of these rabbits did not show a significant increase in the number of circulating monocytes nor was the total number of leucocytes increased.

TABLE 2.

Time of count	Total leucocytes*	Lymphocytes*		Polymorphonuclear cells*		Monocytes*	
		Percentage	Absolute	Percentage	Absolute	Percentage	Absolute
Before injection	11,400	71	8,100	25	2,800	4	500
After 24 hours	10,600	54	5,700	42	4,500	4	400
After 48 hours	15,700	41	7,200	46	6,400	13	2,100
After 72 hours†	21,000	18	3,800	44	9,300	38	8,000

* per cubic millimetre. † animal died at 80 hours.

Results of leucocyte counts on a rabbit after injection of 0·1 ml. of a spore suspension intravenously.

With guinea-pigs differential leucocyte counts were performed on blood from the ball of the foot. Of the 18 guinea-pigs referred to in Table 1, only a few were examined haematologically, and at some stage all showed a significant increase in the number of circulating monocytes. The results are shown in Table 3.

TABLE 3.

Time of count after injection	Guinea-pig number*	Percentage		
		Lymphocytes	Polymorphonuclear cells	Monocytes
6 days	2	10	73	17
6 days	3	21	64	15
14 days	6	13	69	18
24 days	10	28	59	13
40 days	14	24	52	24

* see Table 1.

The monocyte response of guinea-pigs after intraperitoneal injection of a spore suspension.

(b) With Administered Fractions.

The total and differential leucocyte counts following the administration of three different fractions are recorded in Table 4. It may be seen that the mycelial extract (which contains protein, polysaccharide and lipoid material) gives a leucopenia followed by a neutrophil leucocytosis, as well as a monocytosis. The pure polysaccharide gives only a leucopenia followed by a leucocytosis and the lipoid only a monocytosis.

The increase in circulating monocytes in the rabbit was not as marked after intraperitoneal inoculation as after intravenous inoculation of the lipoid.

TABLE 4.

Material injected	Time of count	Total leucocytes*	Lymphocytes*		Polymorphonuclear cells*		Monocytes*	
			p.c.	Absolute	p.c.	Absolute	p.c.	Absolute
Mycelial extract† 2·0 ml.	before injection	8,800	60	5,300	32	2,800	8	700
	after 3 hours	3,000	72	2,100	26	800	2	100
	after 24 hours	25,200	29	7,300	65	16,400	6	1,500
	after 48 hours	22,800	25	5,700	65	14,800	10	2,300
	after 72 hours	16,000	30	4,800	55	8,800	15	2,400
	after 96 hours	14,600	42	6,100	40	5,900	18	2,600
Polysaccharide 20 mg./2 ml. saline	before injection	10,000	48	4,800	44	4,400	8	800
	after 2 hours	7,200	79	5,700	16	1,100	5	400
	after 24 hours	26,600	25	6,700	69	18,300	6	1,600
	after 48 hours	16,200	34	5,500	61	9,900	5	800
	after 72 hours	12,000	42	5,000	54	6,500	4	500
Lipoid 20 mg./2 ml. saline	before injection	11,600	55	6,400	40	4,600	5	600
	after 3 hours	12,800	62	7,900	34	4,400	4	500
	after 24 hours	15,200	48	7,300	45	6,800	7	1,100
	after 48 hours	16,100	32	5,200	50	8,000	18	2,900
	after 72 hours	15,500	24	3,700	48	7,400	28	4,400

* Per cubic millimetre. † This rabbit showed an increase in the eosinophil count from 1 p.c. to 12 p.c. in 96 hours.

The Production of Tubercles by the Lipoid Fraction.

The tubercle producing capacity of the lipoid extract was tested out only by intraperitoneal inoculation of guinea-pigs. Six guinea-pigs were each inoculated by the intraperitoneal route, with 15 mg. lipoid in 1·5 ml. saline. After 48 hours another 15 mg. was administered to each pig. On the fourth day after the first injection one guinea-pig was killed and *post-mortem* examination revealed numerous tubercles about 1 to 2 mm. in diameter and a few larger tubercles in the omentum. There were no adhesions. The picture was much the same in each of the remaining guinea-pigs. Pigs were killed and examined on the 5th, 6th, 7th, 8th and 9th day after injection. In each case material was set aside in 10 p.c. formol-saline for sectioning. Microscopic examination of sections stained with haematoxylin-eosin revealed that the tubercles produced by the intraperitoneal injection of lipoid resembled the lesions produced by infection with the viable organism. There were, of course, no mycelia present, and there was less fibro-cellular tissue. Giant cells were not detectable in tubercles less than seven days old, but appeared in older lesions.

The Serological Behaviour of Fractions.

The polysaccharide fraction was antigenic, stimulating the production of antibodies in the rabbit. Positive precipitation and ring tests were obtained with antiserum prepared against the mycelial extract or the polysaccharide as well as with sera from animals which survived infection.

The haemagglutination titre of the serum when tested against polysaccharide-treated sheep red cells (method to be published later) was forty times higher than the titre obtained in the usual precipitin tests.

Two rabbits immunized against the polysaccharide were given intravenous injections of the spore suspension. Inocula of this size had invariably proved fatal for rabbits within 7 days. These two rabbits, however, appeared to be unaffected when observed for 52 days. A twenty fold increase in the size of the inoculum was made. One rabbit died on the fifth day and the other on the seventh day after the second inoculation. *Post-mortem* examination revealed characteristic lesions.

The lipoid *per se* was non-antigenic but possessed strong adjuvant properties (see Freund *et al.*, 1944, 1948) when mixed with the polysaccharide and injected intradermally. In this respect it is similar to the MPA of *Listeria monocytogenes* (Stanley, 1949).

Intradermal injection of 1 mg. of the polysaccharide into normal guinea-pigs and rabbits caused no reaction. When the same amount of polysaccharide was injected intradermally into animals infected with the aspergillus and containing tubercles in the peritoneal cavity or elsewhere an area of erythema appeared in 24 hours and reached a maximum in 48 hours (see Table 1). This eventually subsided, leaving, after five days, a small hard lump. Positive skin tests were also obtained in animals immunized against the mycelial extract and the polysaccharide but not in animals containing tubercles that had been produced by intraperitoneal inoculation of lipoid. Intradermal injection of the mycelial extract gave a response which was identical with that of the polysaccharide.

Other Biological Properties of Fractions.

Shwartzman reaction. The polysaccharide gave a negative Shwartzman reaction when tested out both in the rabbit and the guinea-pig.

Toxicity. Apart from the effects on the haemopoietic system and the erythema produced in the skin of sensitive animals, the polysaccharide showed no toxic effects. As much as 20 mg.

has been given intravenously to rabbits in one dose. Although a leucopenia was produced, blood sugar estimations were not carried out to determine whether the polysaccharide affected the carbohydrate metabolism of the host. Continued administration of the mycelial extract into the peritoneal cavity of guinea-pigs did not produce adhesions and no animal receiving any fraction developed locomotor ataxia.

DISCUSSION.

It is apparent that the fate of an animal to administration of aspergillus spores depends to a large extent on the route of administration. A mortality rate of 100 p.c. was obtained by intravenous inoculation of 12 animals (3 rabbits, 2 guinea-pigs, 6 mice and 1 pigeon). A mortality rate of 17 p.c. was obtained by the intraperitoneal inoculation of 18 guinea-pigs, although infection occurred (with the production of tubercles or adhesions in 100 p.c. of animals). No evidence of infection was obtained in pigeons, rabbits, guinea-pigs, and mice after intranasal administration of the spores.

Tubercle formation is a characteristic response to infection with most strains of pathogenic aspergilli. It was particularly marked with the strain of *Aspergillus fumigatus* isolated from the penguin, even in the early stages of the disease. The constant findings with this particular strain were ataxia and adhesions in the peritoneal cavity. Adhesions between the lung and the diaphragmatic pleura were observed by Cooper (1946) in a human case of aspergillosis. These toxic effects could not be reproduced by the mycelial extract, polysaccharide or lipoid. These findings are therefore in contrast to those of Henrici (1939) who described the preparation and toxic properties of a substance resembling an "endotoxin" present in "cell sap." The most striking lesions caused by Henrici's preparation were massive haemorrhages in the lungs. Apart from the fact that the strain of *Aspergillus fumigatus* used by Henrici was different from that used in the investigations described here, his "cell sap" was obtained from mincing a mycelial felt obtained by growing the mould on the surface of a liquid medium. This felt contained numerous spores and consequently the extract obtained would contain material from the spores. The mycelia used in the experiments here were free from spores. The use of *mycelia* alone for extraction of material was decided upon because microscopic examination showed that tubercles consisted of a mass of mycelia and only on rare occasions were spores seen *in vivo*.

It has been possible to reproduce the tubercle, characteristic of infection with *Aspergillus fumigatus*, by the administration of sterile lipoid material obtained from the mycelium. This observation is of interest because the mechanism of tubercle formation by non-acid fast organisms (*Listeria monocytogenes* and *Aspergillus fumigatus*) has not yet been elucidated. The compre-

hensive studies of Sabin and her colleagues and of Anderson (Anderson and Chargaff, 1929; Sabin *et al.*, 1930; Sabin, 1932; Anderson, 1932, etc.), have shown that lipoid material (phosphatide fraction and phthioic acid) from *Myco. tuberculosis*, as well as eliciting a monocytic response, can reproduce the epithelioid cell, the giant cell and the tubercle, characteristic of infection with *Myco. tuberculosis*. Some of these characteristics are shared by lipoid material from *Aspergillus fumigatus* and *Listeria monocytogenes* and the relationship between these substances and the bearing they have on experimental tuberculosis will be the subject of a further paper and will not be discussed here.

The polysaccharide fraction is antigenic but incapable of conferring an immunity sufficient to protect animals from large intravenous doses of spores. It is similar to certain bacterial polysaccharides in its ability to cause a leucopenia followed by a neutrophil leucocytosis after intravenous administration, but differs from some of these in being non-toxic. The area of erythema following intradermal inoculation of small quantities serves as a useful indication of infection, and is probably due to the presence of circulating antibody. The test became positive usually about the fifth day after infection and remained so for seven weeks, when the experiment was discontinued.

The adjuvant properties of the lipoid were quite marked and further investigations have been carried out with it. They are not reported in this paper.

SUMMARY.

The condition of experimental aspergillosis in laboratory animals is discussed from observations carried out using a strain of *Aspergillus fumigatus* isolated from a penguin. The disease was characterized by a monocytosis, ataxia, adhesions in the peritoneal cavity and tubercle formation. Examination of a crude mycelial extract, a pure polysaccharide and an acetone-ether-soluble lipoid revealed that the ataxia and the adhesions could not be reproduced by inoculation with any of these products. On the other hand, it was shown that inoculation with the lipoid fraction caused tubercle formation and a monocytosis. The lipoid also possessed adjuvant properties. The polysaccharide fraction was antigenic and of low toxicity. It produced an area of erythema in the skin of immune animals or animals at the later stage of the disease when tubercles and/or adhesions were present.

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THE AUGMENTING ACTION OF LECITHIN AND THE LIPOIDS OF *ASPERGILLUS FUMIGATUS* AND *LISTERIA MONOCYTOGENES* IN ANTIBODY PRODUCTION USING *SALMONELLA TYPHI-MURIUM* AS AN ANTIGEN

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Several hypotheses have been offered to explain the augmenting action of various lipoids and oils, sometimes referred to as "adjuvants," on antibody production. Freund and his colleagues (Freund *et al.*, 1937; Casals and Freund, 1939; Freund *et al.*, 1940; Freund and Opie, 1938; Freund and Bonanto, 1944, and Freund *et al.*, 1948) observed that a close relationship existed between the amount of adjuvant injected, the size of the local lesion and the antibody response. They demonstrated that local lesions obtained with killed tubercle bacilli and paraffin oil contained large mononuclear cells, lymphocytes, numerous polymorphonuclear cells and a few giant cells. These investigators were of the opinion that the oil acted by retarding the distribution of the antigen, but they also mentioned the possibility of the increase in antibody concentration being due to a monocytic reaction.

Similar augmenting properties were demonstrable when influenza virus was used as an antigen (Friedewald, 1944 a, b; Henle and Henle, 1945). Friedewald was of the opinion that "the function of the paraffin oil and acid-fast bacteria is probably to set up a reactive tissue wall about the inoculum and thus localize and maintain the antigenic material at the inoculation site." Friedewald also admitted the probability of the mononuclear cells being involved. The findings of Freund *et al.*, and Friedewald have been extended and confirmed by Ehrich *et al.* (1948), using *Shigella paradysenteriae* (Flexner) as an antigen. By studying the reactions at the site of the injection, these investigators came to the conclusion that the lymphocytic theory (Ehrich and Harris, 1945) fitted their observations better than the reticulo-endothelial theory of antibody formation. An interesting observation was that the lymphocytic reaction of the lymph nodes ran parallel to the antibody response, whereas the monocytic reaction at the injection site was not correlated with this response but reached its peak *after* the maximum antibody production had been reached. On the other hand Sabin (1939) using a red dye-protein antigen, inferred that cells of the reticulo-endothelial system may synthesize antibody protein by modifying the synthesis of cell cytoplasm under the influence of an antigen.

Although the evidence for the presence of antibody in or on lymphocytes is good, it is not extensive (McMaster and Hudack, 1935; Ehrich and Harris, 1942; Dougherty *et al.*, 1944 a, b; White and Dougherty, 1945; Harris *et al.*, 1945; Ehrich and Harris, 1945), and the studies of Hektoen (1911) with dogs and the more recent investigations of Fagraeus (1948) with rabbits and Craddock *et al.* (1949), with cats do not support such findings. The latter workers could find no evidence which indicated lymphocytic transport of antibody to the blood of cats, nor was any significant alteration in the antibody content of cell-free lymph fluid observed after the administration of large doses of adrenal cortical hormones (c.f. Dougherty *et al.*, 1944).

If the observations cited are true, it is apparent that antibodies may be produced by a variety of cells and only further investigation will indicate the relative importance of each type. The majority of workers who have studied the augmenting action of lipoids tend to the view that (1) increased antibody production with lipoid is due to the *slow* liberation of the antigen from the injection site; (2) the monocyte *may* play some part; (3) it is necessary for the vaccine and lipoid to be inoculated together intradermally or subcutaneously.

The experiments described in this paper concern the augmenting properties of certain microbial lipoids that are characterized by producing a marked monocytic reaction in experimental animals. Antibody concentration following the intravenous administration of vaccine and lipoid, either separately or in combination, has been investigated.

Materials and Methods.

Vaccine. A recently isolated strain of *S. typhi-murium* was grown on nutrient agar for 18 hours and the growth washed off in saline. The organisms were killed by heat at 55° C. for 30 minutes and the vaccine kept at a concentration of 80,000 × 10^6 organisms/ml. at 4° C.

Lipoids. These were prepared by methods described in previous communications (Stanley, 1949). The acetone-ether soluble fractions were kept in diethyl ether. For use, saline suspensions were obtained by distilling, *in vacuo*, the ethereal solution in the presence of 0·85 p.c. NaCl containing 0·03 p.c. sodium lauryl sulphate. The final concentration of lipoid material was 10 mg./ml. The solutions were warmed and thoroughly shaken before use. All injections were made with a tuberculin syringe. The lecithin used in one of the experiments was that of Schering-Kahlbaum, and was prepared from eggs.

EXPERIMENTAL.

In preliminary experiments with lipoid material from *A. fumigatus* and *List. monocytogenes* together with heavy mineral oil, and with the antigens *S. typhi-murium* vaccine, *A. fumigatus* polysaccharide or *List. monocytogenes* vaccine, it was shown that after intradermal inoculation of rabbits, the lipoids behaved as adjuvants after the manner described by Freund *et al.* However, in the experiments to be described here, heavy mineral oil and other substances likely to make the reaction more complex were omitted. As it seemed likely that the monocyte played some part in the augmenting action of antibody production it was

decided to study the antibody response to the vaccine in animals receiving injections of monocyte-producing substances by different routes, either alone or in combination with the vaccine. In this respect it became of interest to determine whether an increased antibody titre could be obtained if the vaccine were given by some route (e.g. intravenously) other than the intradermal or subcutaneous one where *slow* liberation of the antigen may occur and is considered one factor responsible for increased antibody titre.

Intradermal Administration of Vaccine Plus Lipoid.

Antibody production in rabbits following the intradermal administration of *S. typhi-murium* vaccine alone and in combination with lipoid from *A. fumigatus, List. monocytogenes* or a diphtheroid, is given in Table 1. It was previously shown that lipoid material from several diphtheroids examined did not stimulate the production of monocytes when injected intravenously into rabbits (unpublished observation). "H" and "O" antibody titres were determined as well as the haemagglutinating titre of the serum using sheep cells treated with *S. typhi-murium* polysaccharide.

TABLE 1.

	Rabbit received	Antibody titre after					
		10 days			20 days		
		"H"	"O"	Haem.†	"H"	"O"	Haem.†
R.13	vaccine alone	40	<40	320	<40	<40	640
R.14	vaccine + lipoid AF*	160	80	1,280	320	80	2,560
R.15	vaccine + lipoid LM*	160	40	1,280	160	80	5,120
R.16	vaccine + lipoid D*	40	40	640	40	<40	320

*AF = *Aspergillus fumigatus.*
LM = *List. monocytogenes.*
D = Diphtheroid.
† = Haemagglutination titre with polysaccharide-treated sheep red cells.

It is evident that the lipoids capable of stimulating the production of monocytes can enhance antibody production, whereas the lipoid of the diphtheroid, which does not act as a monocyte-producing agent, does not enhance antibody production. A further experiment using lipoid of *List. monocytogenes* was conducted in guinea-pigs. Four guinea-pigs were inoculated subcutaneously, two with vaccine alone and two with vaccine and lipoid mixed prior to administration. Five days after the last injection of 0·2 ml. of a suspension containing 200 × 10^6 organisms/ml., given on 3 occasions at 3 day intervals, the animals' sera were examined for antibodies. The "H" and "O" titres are recorded in Table 2.

TABLE 2.

Guinea-pig number	Guinea-pig received subcutaneously	Antibody titre five days after last injection	
		"H"	"O"
P.41	vaccine	8	tr 4
P.42	vaccine	8	<4
P.43	vaccine + lipoid*	512	128
P.44	vaccine + lipoid*	1,024	128

* Lipoid from *List. monocytogenes.*

Intravenous Administration of Vaccine Plus Lipoid.

This experiment was performed to ascertain if an increased number of circulating monocytes was associated with increased antibody titre following the intravenous administration of vaccine plus lipoid. The rabbits were divided into two groups. The first group comprised four rabbits, each of which received four injections with a three day interval between each injection. The two rabbits (R18 and R19) used as controls received vaccine alone. The test animals (R20 and R21) received a lipoid-vaccine suspension that had been prepared 48 hours before the first injection. The lipoid was that of *List. monocytogenes.* The results recorded in Table 3 show that the presence of lipoid in the inoculum caused a definite increase in "O" antibody titre but no significant difference was observed in "H" antibody titre. The animals receiving lipoid developed a monocytosis 48 hours after inoculation.

TABLE 3.

Inoculum	Rabbit number	Antibody titre on 16th day	
		"H"	"O"
* Vaccine	R.18	40,000	256
	R.19	20,000	128
* Vaccine + lipoid	R.20	20,000	1,024
	R.21	40,000	1,024

* The following inocula were given at 3-day intervals:

0·1 ml. of suspension containing 100 × 10^6 orgs./ml.
0·3 ml. of suspension containing 100 × 10^6 orgs./ml.
0·6 ml. of suspension containing 100 × 10^6 orgs./ml.
0·1 ml. of suspension containing 500 × 10^6 orgs./ml.

The second group consisted of eight rabbits each receiving 0·2 ml of vaccine containing 500 × 10^6 organisms/ml. In this experiment the vaccine and the lipoid were not mixed prior to injection, but were inoculated separately. Two rabbits (R24 and R25) received vaccine alone. Three rabbits (R26, R27 and R28) received 15 mg. of lipoid intravenously (in the left ear) at the same time as the vaccine was administered in the right. The remaining rabbits (R29, R30 and R31), received 20 mg. lipoid by the intraperitoneal route at the same time as the vaccine was given intravenously. The rabbits receiving lipoid were each given two further injections of the same material with an interval of three days between each injection. Total and differential leucocyte counts were performed at two or three day intervals throughout the course of this experiment and revealed that animals receiving lipoid from *A. fumigatus* or *List. moncytogenes* maintained a high monocyte count for approximately 12 days, while animals receiving lipoid from the diphtheroid showed a monocyte count that fell within normal limits. The monocyte count of most animals was within normal limits when the highest antibody titres were recorded between the 16th and 19th days. The results of antibody estimations are given in Table 4.

Intravenous Administration of Vaccine Plus Lecithin.

Doane and Moore (1931) observed that lecithin (prepared from brain) was the only substance of those tested that behaved, on injection into animals, in the same way as tuberculophosphatide. In other words lecithin was capable of stimulating the production of monocytes and could ultimately cause the formation of tubercles. In these respects it was similar to the lipoids of *List. monocytogenes* and *A. fumigatus* (Stanley, 1949 and unpublished observations).

TABLE 4.

Animal number	Rabbit received			Antibody titre after											
				5 days			13 days			19 days			26 days		
	intravenously (right ear)	intravenously (left ear)	by the intraperitoneal route	"H"	"O"	Haem.*	"H"	"O"	Haem.*	"H"	"O"	Haem.*	"H"	"O"	Haem.*
R.24	vaccine	—	—	1,280	20		5,120	80	320	5,120	160	640	80	40	640
R.25	vaccine	—	—	1,280	40		5,120	160	320	2,560	160	1,280	80	40	1,280
R.26	vaccine	lipoid (AF)†	—	1,280	40		10,240	1,280	2,560	10,240	640	10,240	160	160	2,560
R.27	vaccine	lipoid (LM)†	—	640	20		10,240	1,280	1,280	10,240	1,280	5,120	640	320	2,560
R.28	vaccine	lipoid (D)†	—	2,560	40		10,240	80	320	5,120	160	1,280	160	40	1,280
R.29	vaccine	—	lipoid (AF)	1,280	80		2,560	2,560	5,120	2,560	1,280	20,480	320	320	10,240
R.30	vaccine	—	lipoid (LM)	2,560	40		5,120	1,280	5,120	5,120	640	20,480	320	160	10,240
R.31	vaccine	—	lipoid (D)	640	20		2,560	40	160	2,560	80	640	80	20	640

*Haemagglutination titre using *S. typhi-murium* polysaccharide treated sheep red cells.

† AF = *Aspergillus fumigatus*. LM = *Listeria monocytogenes*. D = Diphtheroid.

It was, therefore, decided to test out the augmenting action of lecithin. Four rabbits were inoculated intravenously with *S. typhi-murium* vaccine, with and without the lecithin which was mixed prior to administration and had a final concentration of 30 mg./ml. The final concentration of organisms in the vaccine was 500 × 10^6/ml., and 0·1 ml. was injected. Total and differential leucocyte counts revealed that a monocytosis occurred in the lecithin-treated rabbits (R36 and R37), but not in the untreated controls (R34 and R35); a neutrophil leucocytosis occurred in all animals 24 to 48 hours after inoculation. The antibody titres five days after administration of vaccine are given in Table 5. Further antibody estimations are not recorded here as one of the animals died on the 6th day.

TABLE 5.

	Rabbit received	Antibody titre after five days		
		"H"	"O"	Haem.
R.34	vaccine + saline	20	<20	640
R.35	vaccine + saline	20	<20	640
R.36	vaccine + lecithin	320	80	5,120
R.37	vaccine + lecithin	160	40	2,560

DISCUSSION.

It is apparent that increased antibody titres may be obtained when a particulate antigen is injected *intravenously* with some microbial lipoids. The augmentation of antibody titre occurs irrespective of whether the lipoid material is mixed with the vaccine prior to injection or given independently by the same or the intraperitoneal route. These few experiments indicate that it is not necessary for an antigen to remain for long periods at the site of inoculation, subcutaneously or intradermally, for increased production with the aid of certain lipoids to be demonstrated.

Of the four lipoids used, only one (from a diphtheroid) failed to augment the antibody titres. This particular lipoid was the only one that failed to cause an increase in the number of circulating monocytes in rabbits. It may thus be argued that the monocyte may play some part in increasing antibody production under these experimental conditions. On the other hand it is possible that some cells other than monocytes were affected, resulting in increased antibody production. For example, the plasma cell, as suggested by Fagraeus (1948), studying the secondary response, may be involved in this reaction, but as biopsy specimens of spleen were not examined, this point is still obscure. Lecithin was found to act in a manner similar to the two monocyte-stimulating microbial lipoids. In no case was a lymphocytosis demonstrable.

Both "H" and "O" antibody titres were increased. Corresponding increases in antibody titre measured by a haemagglutination method using polysaccharide-treated sheep cells indicated that this was a suitable if not a more sensitive method for measuring antibody content than the agglutination reaction.

SUMMARY.

Using *S. typhi-murium* vaccine as antigen it was demonstrated that lipoid material from *Listeria monocytogenes* or *Aspergillus fumigatus* could act as "adjuvants" in antibody production after intradermal inoculation similar to the manner described by Freund. It was further shown that increased antibody titre resulted if the vaccine was given intravenously (1) when the lipoid was mixed with vaccine prior to administration or (2) given independently by the intravenous or intraperitoneal route. It was suggested that the augmentation of antibody titre may be associated in some way with the stimulation of monocytes as lipoid material incapable of increasing the number of circulating monocytes failed to augment antibody titre. Lecithin, the only non-microbial lipoid tested stimulated both the production of monocytes and antibodies.

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STUDIES ON *LISTERIA MONOCYTOGENES*

III. THE FAILURE TO ISOLATE THE ORGANISM FROM THE HUMAN THROAT

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In a previous communication (Stanley, 1949) it was shown that *Listeria monocytogenes* could not be isolated from the blood of twenty patients diagnosed clinically and haematologically as infectious mononucleosis although agglutinins to that organism were demonstrable in 35 p.c. of cases. As nothing has previously been recorded concerning the carrier-rate or incidence of this organism an interpretation of the above results is difficult. Webb (1943) suggested that the search for listeria should include cultures of the enlarged superficial lymph glands and cited the six cases of Baldridge *et al.* (1926), as an example of such an investigation. As the "diphtheroids" isolated by these investigators were discarded as contaminants their identity with *Listeria monocytogenes* was not established. Webb (1943) obtained negative results with cultures from two axillary lymph nodes.

Because of the lack of information on the occurrence of listeria in the throat and nasopharynx the following experiments were carried out.

Materials and Methods.

A total number of 3,558 swabbings taken from the nose, throat or nasopharynx of patients admitted to the Prince Henry Hospital between September 1948 and the end of March 1949, was examined for the presence of listeria. The swabbings were collected from those patients admitted with a provisional diagnosis of diphtheria, scarlet fever, acute tonsillitis, cervical adenitis or infectious mononucleosis.

Listeria monocytogenes is as resistant to the action of potassium tellurite as members of the genus *Corynebacterium*. Since the organisms have, on more than one occasion, been confused with corynebacteria, diphtheroids growing on the "tellurite plates" used for the routine isolation of *C. diphtheriae* were examined to determine if they possessed any antigenic relationship with an Australian strain of *Listeria monocytogenes* (Stanley, 1948). Suitable colonies were inoculated onto Loeffler serum slopes which were incubated at 37° C. for 24 hours. The resulting growth was washed off in 1·0 ml. of 5 p.c. sodium chloride solution and allowed to stand for periods varying from 30 minutes to two hours. Slide agglutinations were then carried out at this concentration of sodium chloride with anti-listeria rabbit serum and normal rabbit serum (control).

All other swabbings received were plated out on blood-agar plates. Colonies resembling listeria were filmed, and, if shown to be Gram-positive bacilli with typical morphology, sub-cultured onto Loeffler serum slopes and treated in the same way as the diphtheroids. Of the 166 cultures isolated from blood plates, 157 were from cases of scarlet fever, 6 were from cases of acute tonsillitis, 2 were from cases of cervical adenitis and 2 from cases of infectious mononucleosis.

RESULTS.

These are given in the form of a Table.

TABLE 1.

	Number of swabbings		Slide agglutination tests with		
	received	selected for examination	5 p.c. NaCl	normal rabbit serum	Listeria antiserum
Diphtheroids	2,556	980 (709)	—	—	—
		980 (65)	+	ND	ND
		980 (206)	—	+	+
Others	1,002	166 (126)	—	—	—
		166 (14)	+	ND	ND
		166 (26)	—	+	+
Total	3,558	1,046			

ND = test not performed.

It may be seen that of the 3,558 cultures selected only 1,046 were considered suitable for further investigation. This occurred because either some of the tellurite cultures were sterile or the blood-agar cultures obviously contained no colony resembling *Listeria monocytogenes*. A result was recorded as positive if agglutination occurred within 20 seconds. Fifteen of the 232 cultures that were sensitive to normal rabbit serum appeared to agglutinate the listeria antiserum slightly more rapidly. These cultures were further investigated when an examination of their cultural and biochemical characteristics was made. Only two (D.6265 and D399) of the fifteen strains showed morphological, cultural and biochemical properties similar to *Listeria monocytogenes*. The two cultures, besides giving a negative conjunctivitis reaction, failed to elicit the monocytosis characteristic of infection with listeria after intravenous inoculation of rabbits. On this evidence it was decided that the cultures were not classical *Listeria monocytogenes*.

DISCUSSION.

The large number (21 p.c.) of strains sensitive to normal rabbit serum may have masked some results not demonstrable by the slide agglutination technique. Stock cultures of listeria are generally agglutinated more rapidly by specific serum than sensitive diphtheroids are by normal rabbit serum. None of eight stock cultures of listeria was sensitive to normal rabbit serum. Although only a few cases of infectious mononucleosis occurred over the six month period covering these examinations, it would seem that the failure to isolate organisms

agglutinating antiserum prepared against the Australian strain, from 3,558 swabbings, indicates that organisms of this antigenic type are rare.

This evidence suggests that *Listeria monocytogenes* may not be an important factor in the etiology of infectious mononucleosis in spite of the evidence obtained by Stanley (1949), in an earlier series of cases, in which 35 p.c. of patients showed the presence of listeria agglutinins to a significant titre. It is possible that the presence of these agglutinins in some cases of infectious mononucleosis may be due to the production of globulin which is unusually reactive and possesses a configuration fitting the antigenic pattern of *Listeria monocytogenes* as well as that of other organisms (e.g. Newcastle Disease virus).

SUMMARY.

An examination of 3,558 swabbings revealed that *Listeria monocytogenes* could not be isolated from the throat, nose or nasopharynx of patients admitted to hospital with diphtheria, scarlet fever, acute tonsillitis, cervical adenitis or infectious mononucleosis.

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THE EFFECT OF AN ACRIDINE DYE—5-AMINOACRIDINE—ON THE METABOLISM OF *STAPHYLOCOCCUS AUREUS*

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Evidence is accumulating that the bacteriostatic and antiseptic action of various chemical substances is due to their interference with enzyme systems necessary for the growth or even life of the organism.

These chemical substances may act on enzyme systems in a variety of ways but, for the present, it seems more important to determine which enzyme systems are affected rather than how this effect is produced.

The acridine dyes are active bacteriostatic agents, and of them, 5-Aminoacridine hydrochloride was chosen as one of the most active members of this group, with the added advantage of giving only a feebly coloured solution. The organism chosen was the common cause of infection, *Staph. aureus.*

Synthetic processes associated with growth can proceed only if the necessary energy is provided, and as carbohydrate metabolism seems to provide this energy most readily the investigation of enzymes concerned in this process was selected as a first approach to the problem. This involved research into the normal metabolism of the staphylococci.

Actively growing bacteria were used to obtain conditions as closely resembling the natural state of the organism as possible.

Materials and Methods.

The organism used was *Staphylococcus aureus*, a stock culture being kept in nutrient broth (Lab. Lemeo 10 p.c. bacteriological peptone 10 p.c., NaCl 10 p.c., pH adjusted to 7·6) and nutrient agar slopes in 150 ml. bottles inoculated 16–18 hours before use. The bacteria were harvested in distilled water, centrifuged, and resuspended in distilled water.

Manometric experiments were carried out in standard Warburg manometers, in air at 37° C.

Glucose estimations. These were carried out according to the Hagedorn and Jensen (1923 a, b) alkaline ferricyanide method.

Hexosemonophosphate (H.M.P.) was prepared according to K. du Bois and V. R. Potter (1943), purity after repurification being 94 p.c.

Hexosediphosphate (H.D.P.) was prepared according to C. Neuberg and H. Lustig (1942) and purified according to K. Bernhauer (1936): purity 85 p.c.

EXPERIMENTAL.

The effect of 5-Aminoacridine in various concentrations on the oxygen uptake of *Staph. aureus* in the presence of culture medium was first determined.

The experiments were carried out manometrically. Main cup: M/15 PO_4 buffer pH 7·6, 0·5 ml.: bacterial suspension 1·0 ml.; nutrient broth 1·0 ml., and 5-Aminoacridine hydrochloride 0·1 ml. to give the final concentrations indicated. Filter paper strips with 20 p.c. KOH 0·15 ml. in centre cup, to absorb CO_2.

TABLE 1.

10^{-4} M 5-Aminoacridine	—	1·65	2·2	3·3	33
μl. O_2 uptake 80 min.	790	641	513	413	40

It is seen that the concentration of 5-Aminoacridine which completely inhibits growth, approximately $3·3 \times 10^{-4}$ M, does not completely inhibit oxygen uptake, so that 5-Aminoacridine inhibits some system essential for growth of the bacteria, but in growth-preventing concentrations does not entirely suppress respiration.

The oxygen uptake by *Staph. aureus* in the presence of various substrates was determined.

Manometric experiments; Main cup: M/15 PO_4 buffer pH 7·6, 0·5 ml.; bacterial suspension 1·0 ml.; 0·3 M substrate 0·1 ml. (or water) tipped in from side cup after equilibration; 20 p.c. KOH 0·15 ml., and filter paper strips in centre cup to absorb CO_2.

TABLE 2.

Substrate	μl. O_2 uptake 1 hr.
—	31
Glucose	260
Na pyruvate	107
Na citrate	43
Na fumarate	40
Na succinate	41
Na acetate	32

Glucose and pyruvate were the only compounds among those tested, to give an appreciable oxygen uptake with staphylococci. The effect of 5-Aminoacridine on the metabolism of these substances was therefore investigated.

Manometric experiment; Main cup: M/15 PO_4 buffer pH 7·6, 0·5 ml.; bacterial suspension 1·0 ml., 0·3 M substrate 0·1 ml. (or water) tipped in from side cup after equilibration. $2·17 \times 10^{-3}$ M 5-Aminoacridine-HCl 0·3 ml. or water. 20 p.c. KOH 0·15 ml. and filter paper strips in centre cup to absorb CO_2.

TABLE 3.

Substrate	5-Aminoacridine	μl. O_2 uptake 1 hr.
Glucose	—	532
Glucose	+	361
Na pyruvate	—	126
Na pyruvate	+	165

The oxygen uptake in the presence of glucose was significantly reduced by 5-Aminoacridine but not that with pyruvate. Glucose was selected as the substrate for further investigation.

As well as reducing the oxygen uptake in the presence of glucose, 5-Aminoacridine also partially inhibits the disappearance of "total reducing substance." The reducing substances were estimated by the Hagedorn and Jensen alkaline ferricyanide method which shows reduction not only with glucose but also with hexosemono and diphosphates although to a lesser extent. The term "total reducing substances" is used to indicate all the reducing substances present expressed as glucose. Over a number of estimations the total disappearance of reducing substance in 5 hours averaged 239 mg. p.c. in the absence of 5-Aminoacridine and 155 mg. p.c. in the presence of $3{\cdot}43 \times 10^{-4}$ M 5-Aminoacridine HCl with glucose as substrate.

As it seemed possible that the glucose metabolism of staphylococci might in part go through hexose phosphate stages, the metabolism of these substances was examined in the presence and absence of 5-Aminoacridine.

Glucose-1-phosphate, Robison ester, and hexosediphosphate were used, and in all experiments where glucose was compared with a hexosephosphate the molar concentrations of the two were the same.

Glucose-1-phosphate was metabolised at a much lower rate than glucose, as measured by oxygen uptake, and 5-Aminoacridine inhibited this by about 20 p.c. more than that of glucose. These results suggest that glucose-1-phosphate does not lie in the direct line of glucose metabolism.

The oxygen uptake with Robison ester was the same as with an equivalent amount of glucose and followed the same time course. The reduction of oxygen uptake caused by 5-Aminoacridine was also the same with Robison ester and glucose. Glucose then is probably, at least in part, phosphorylated to Robison ester in its metabolism by staphylococci.

Hexosediphosphate gives a slower oxygen uptake than glucose, but this uptake is little or not at all affected by 5-Aminoacridine. When very actively metabolising suspensions of staphylococci were used, 5-Aminoacridine had no effect, but when metabolism was sluggish there was a slight reduction in oxygen uptake in the presence of the drug. The slower oxygen uptake given with hexosediphosphate may be due to a slower absorption by the bacteria than with glucose.

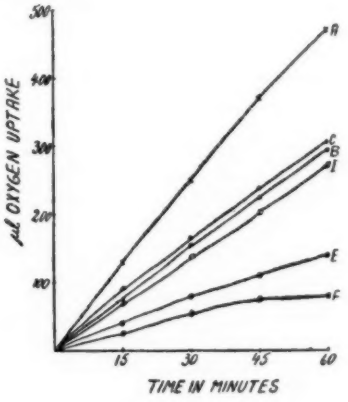

Fig. 1. Effect of 5-Aminoacridine on oxygen uptake with glucose and hexosephosphate.

Manometric experiments; M/15 PO_4 buffer pH 7·6, 0·5 ml.; bacterial suspension 1·0 ml.; 0·3 M substrate 0·1 ml. tipped in after equilibration; $2{\cdot}17 \times 10^{-3}$ M 5-Aminoacridine hydrochloride, 0·3 ml. (or water); 20 p.c. KOH and filter paper strips in centre cup to absorb CO_2.

A. Glucose or Robison ester. B. Glucose or Robison ester with 5-Aminoacridine. C. Hexosediphosphate. D. Hexosediphosphate with 5-Aminoacridine. E. Glucose-1-phosphate. F. Glucose-1-phosphate with 5-Aminoacridine.

These results suggest that, in part at any rate, glucose metabolism with staphylococci goes through the hexose phosphate stages, and that 5-Aminoacridine inhibits phosphohexokinase.

To elucidate this suggestion further, the change in inorganic phosphate in the incubation mixture after a 5 hour period of incubation was determined. With glucose there was a slight

reduction of phosphate of 1·7 mg. p.c. to 3·2 mg. p.c. 5-Aminoacridine did not affect this slight reduction suggesting that it has no effect on the primary phosphorylisation of glucose.

With Robison ester, there was an increase in inorganic phosphate averaging 11·3 mg. p.c. in 5 hours; 5-Aminoacridine had a marked effect there, the average increase of inorganic phosphate in the presence of a $3{\cdot}43 \times 10^{-4}$ M dilution of the drug falling to 3·2 mg. p.c. The explanation for this is not obvious.

The "total reducing substance" disappearance with Robison ester was much less than with glucose, averaging 155 mg. p.c. as compared with 239 mg. p.c. in the presence of glucose.

This suggests that a part of the glucose metabolism does not go through the Robison ester stage. As was shown earlier 5-Aminoacridine in amounts which completely inhibit growth of staphylococci only partially inhibits glucose utilization. It is possible that in the presence of 5-Aminoacridine more of the glucose follows another path not affected by the drug.

The results discussed above suggest that glucose, in part, goes through a phosphate cycle, and that 5-Aminoacridine inhibits the conversion of hexosemonophosphate to hexosediphosphate. This part would seem to be related to the growth processes of the organism.

Glucose Disappearance in Presence of Amino Acids.

Since 5-Aminoacridine inhibits growth of bacteria, and amino acids are necessary for protein synthesis, the effects of amino acids on glucose utilisation were investigated. The first step was to determine the response with a mixture of amino acids, this latter containing most of the amino acids specified by E. H. Frieden and C. N. Frazer (1947) as necessary for optimum growth of *Staph. aureus*, in the relative amounts recommended.

List of amino acids: Glycine $1{\cdot}52\times10^{-4}$ M; dl-valine $20{\cdot}56\times10^{-4}$ M; l-leucine $21{\cdot}5\times10^{-4}$ M; dl-aspartic acid $8{\cdot}57\times10^{-4}$ M; l-glutamic acid $43{\cdot}54\times10^{-4}$ M; dl-threonine $10{\cdot}1\times10^{-4}$ M; l-proline $21{\cdot}0\times10^{-4}$ M; dl-methionine $8{\cdot}05\times10^{-4}$ M; dl-phenylalanine $7{\cdot}27\times10^{-4}$ M; l-tyrosine $11{\cdot}0\times10^{-4}$ M; l-arginine HCl $7{\cdot}0\times10^{-4}$ M; l-lysine HCl $4{\cdot}23\times10^{-4}$ M; l-histidine HCl $12{\cdot}57\times10^{-4}$ M. The amino acids were dissolved in distilled water and brought to pH 7·6.

Experiments were carried out in 1 inch pyrex test tubes stoppered with cotton wool and shaken at 37° C.

Incubation mixture. Bacterial suspension, 2·0 ml.; M/15 PO_4 buffer pH 7·6, 1·0 ml.; 0·3 M glucose, 0·2 ml.

Additions. Amino acid mixture, 0·6 ml., final conc. as indicated above. $2{\cdot}17\times10^{-3}$ M 5-Aminoacridine HCl, 0·6 ml.

TABLE 4.

Additions	mg. p.c. "total reducing substance" disappearing in 165 min.
—	98
5-Aminoacridine	36
Amino acid mixture	164
Amino acid mixture + 5-Aminoacridine	57

The presence of a mixture of amino acids significantly increased the amount of "total reducing substance" disappearing. This increase in glucose utilisation is inhibited by 5-Aminoacridine.

A similar experiment was performed measuring oxygen uptake manometrically. Main cup: M/15 PO_4 buffer pH 7·6, 0·5 ml.; bacterial suspension, 1·0 ml.; amino acid mixture or water, 0·3 ml.; 0·3 M glucose 0·1 ml. or water tipped in from side cup after equilibration; fluted filter papers with 0·15 ml. 20 p.c. KOH in centre cup to absorb CO_2.

TABLE 5.

Additions	µl. O_2 uptake 90 min.
Glucose	609
Amino acid mixture	849
Amino acid mixture + glucose	968

The oxygen uptake does not give a good indication of added glucose disappearance as the effect is merely additive. It would appear that in the presence of glucose, oxygen uptake due to amino acid metabolism is largely suppressed.

Glutamic and aspartic acids were the only amino acids to have an increasing effect on glucose metabolism either when the other amino acids were used singly or in various mixtures. Quite small amounts of glutamic and aspartic acids increased the glucose disappearance.

TABLE 6.

General conditions as for Table 4.

Additions (10–4 M)	mg. p.c. "total reducing substance" used in 4¾ hours
50·34 glutamic acid	246
25·16 glutamic acid	254
12·58 glutamic acid	236
6·29 glutamic acid	226
55·64 aspartic acid	264
27·82 aspartic acid	227
13·91 aspartic acid	251
6·95 aspartic acid	238
—	178

In an attempt to elucidate the effect of glutamic acid on glucose utilization, McIlwain's (1939), (1943), (1946 a), (1946 b) work on the production of glutamine from glutamic acid by glycolysing streptococci was considered. Attempts were made to demonstrate the production of glutamine from glutamic acid in glycolising mixtures of *Staph. aureus* using the method of W. H. Elliott (1948) to trap any glutamine with hydroxylamine hydrochloride and develop a colour by adding 1:1 hydrochloric acid, trichloracetic acid and 10 p.c. $FeCl_3$ $6H_2O$ in 0·2 M HCl at the termination of the experiment. No appreciable colour developed, but on estimating the amount of glucose used the following results were obtained.

Incubation Mixture: 0·1 M acetate buffer pH 5·4, 1·0 ml.; bacterial suspension, 1·0 ml.; M/15 phosphate, 0·5 ml.; 0·3 M glucose, 0·2 ml.

Additions: 0·066 M hydroxylamine HCl pH 5·4, 0·5 ml.; 0·06 M glutamate pH 5·4, 0·5 ml.

TABLE 7.

Additions	mg. p.c. "total reducing substance" used in 3½ hours
——	96
Glutamate	138
Hydroxylamine HCl	109
Hydroxylamine HCl + glutamate	95

It is seen that hydroxylamine inhibits the effect of glutamic acid on glucose disappearance while it leaves glucose disappearance in the absence of glutamic acid unaffected. McIlwain was unable to demonstrate the production of glutamine by the Elliott technique using whole streptococci. It appears that either glutamine is formed by the intact *Staph. aureus* but being within the bacterial cell cannot be demonstrated by the technique used or that hydroxylamine unites with some other product formed from glutamic acid, necessary to bring about the accelerating effect on glucose disappearance. Whatever this substance is, 5-Aminoacridine inhibits its effect.

In an attempt to determine the effect of glucose on glutamic acid disappearance the following experiment was carried out.

The previous incubation mixture at pH 7·6 was used. At the termination of incubation, the volume was reduced on a boiling water bath, and spots applied to filter paper strips (Whatman No. 1). These were treated according to the paper chromatography method using water saturated phenol as solvent, and ninhydrin in butyl alcohol to develop colour.

In the absence of glucose all added glutamic acid (64 mg. p.c.) had disappeared in 4½ hours.

5-Aminoacridine inhibited this complete disappearance and easily detectable amounts of glutamic acid remained. In the presence of glucose not all the glutamic acid had disappeared whether 5-Aminoacridine was present or not. It appears that in the presence of glucose only part of the glutamic acid is broken down and utilized, producing the effect of added disappearance of "total reducing substance"; and for the rest, glucose is attacked in preference to glutamic acid.

Since aspartic acid has a similar effect to glutamic acid on glucose utilization it seems probable that α ketoglutaric and oxalacetic acids may be formed from these amino acids and act as catalysts in the tricarboxylic acid cycle or some part of it. 5-Aminoacridine may prevent the production of these acids or may interfere in some enzymatic processes associated with these acids. The latter does not seem very likely in view of the fact that oxygen uptake due to pyruvate as substrate is not inhibited by 5-Aminoacridine.

McIlwain found with *Strep. haemolyticus* that glutamine stimulated glycolysis while glutamic or α ketoglutaric acids did not. He found a stimulation of glycolysis coupled with the conversion of glutamine to glutamic acid and ammonia. Apparently conditions are quite different with the strain of *Staph. aureus* used in these experiments, and the production of glutamine may not be the important factor with this organism.

SUMMARY.

The effect of 5-Aminoacridine on the oxygen uptake of *Staphylococcus aureus* with glucose, glucose-1-phosphate, Robison ester and hexosediphosphate as substrates was investigated.

Some data are given on the change in inorganic phosphate with glucose or Robison ester as substrate, in the presence and absence of 5-Aminoacridine.

The influence of amino acids, more especially glutamic and aspartic acids on utilization of glucose by *Staphylococcus aureus* was determined, as also the effect of 5-Aminoacridine on the above.

Hydroxylamine hydrochloride was found to affect the utilization of glucose by the organism in the presence of glutamic acid.

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